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Modern methods for the synthesis of peptide – oligonucleotide conjugates †

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Abstract. The published data on the methods of chemical solution and solid-phase synthesis of peptide – oligonucleotide conjugates are reviewed. The known methods are systematised and their advantages and disadvantages are considered. The approaches to the solution synthesis of peptide – oligonucleotide conjugates are systematised according to the type of chemical bonds between the fragments, whereas those to the solid-phase synthesis are classified according to the procedure used for the preparation of conjugates, *viz*., stepwise elongation of oligonucleotide and peptide chains on the same polymeric support or solid-phase condensation of two presynthesised fragments. The bibliography includes 141 references.

I. Introduction

Complexes of nucleic acids with proteins play an important role in the storage and transmission of genetic information. These supramolecular structures include ribosomes, chromatin, viruses and complexes of nucleic acids with enzymes and regulatory proteins. It is of note that these structures maintain their stability by virtue of protein – nucleic acid (NA) non-covalent interactions.

Protein – NA complexes the components of which are linked by covalent bonds have been known since long ago [1–3] and became the subject for detailed investigations aimed at elucidation of their chemical structures and functions in the cell. The most convenient tools for the study of such complexes are model peptide – nucleotide conjugates.

The first peptide – oligonucleotide conjugates with phosphoramide, phosphodiester, amide and ester bonds between the fragments were synthesised by researchers at the Department of Chemistry of M V Lomonosov Moscow State University led by M A Prokof'ev and Z A Shabarova.[3,4] In the late 1960's – early 1970's, these scientists performed the synthesis of the first oligonucleotide peptides, *viz*., mixed biopolymers containing oligonucleotide and peptide fragments.[5,6] However, at that time these studies were not extended further, primarily because of the lack of clearcut definitions of molecular-biological tasks which might be solved through the use of peptide – oligonucleotide conjugates.

The situation changed dramatically in the mid-1980's when it was first suggested to improve the intracellular transport of antisense oligonucleotides through their covalent attachment to various peptides able to penetrate the cell membrane. Noteworthy, the interest in peptide – oligonucleotide conjugates arose simultaneously with notable improvement of existing techniques for the formation of peptide and internucleotide bonds and the development of the solid-phase methodologies which eventually led to complete automation of oligonucleotide and peptide synthesis.

A vast number of papers on peptide – oligonucleotide conjugates published in the last 10 – 15 years brought forth the necessity to systematise the existing techniques of their synthesis. Previous studies on the synthesis of peptide – oligonucleotide conjugates were briefly described in the reviews [7–10] published in the early 1990's and devoted to a broader field, *viz*., the synthesis of oligonucleotide derivatives.

The present review includes two main sections describing the synthesis of peptide – oligonucleotide conjugates in solution and on insoluble solid supports. The methods for the preparation of oligonucleotide conjugates with amino acids and proteins fell outside the scope of the review, since each of these groups of methods is a subject for independent reviews.

In considering the methods for the synthesis of peptide – oligonucleotide conjugates in solution, the main criterion for the systematisation of the published data was the type of the chemical bond between the two components of the conjugate molecule, while the procedure for the preparation of conjugates (stepwise elongation of oligonucleotide and peptide chains on the same polymeric support or solid-phase condensation of two presynthesised fragments) was used as a criterion of choice in the analysis of solid-phase methods.

Here we do not touch upon the problems relevant to the structures of the intermediate and target compounds, for in the absolute majority of studies correct analysis has been carried out and the most advanced physicochemical approaches, particularly, standard procedures for the isolation of oligonucleotides and

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† This review is dedicated to the memory of M A Prokof'ev and Z A Shabarova, Professors of M V Lomonosov Moscow State University and outstanding pedagogues and scientists who have laid the groundwork for the synthesis of compounds considered.

peptide – oligonucleotide conjugates have been employed. Nor did we consider the prospects of the application of peptide – oligonucleotide conjugates in antisense biotechnology, because these problems were analysed in the reviews published by Gait *et al.*[11] and Tung and Stein [12] in 2000. The latter publication describes the preparation of peptide – oligonucleotide conjugates, albeit in a very concise form, without going into details about the chemical reactions involved in the synthesis of hybrid molecules.

Such a limitation of the range of problems under consideration has made it possible to focus the main attention on the description of various approaches to the synthesis of peptide – oligonucleotide conjugates and the formation of covalent bonds between the oligonucleotide and peptide fragments of the conjugates and to perform a detailed analysis of the problems involved.

The principles underlying the classification and nomenclature of peptide – oligonucleotide conjugates have not been finally established yet. There exist several types of naming for the peptide – oligonucleotide conjugates. Some authors propose to consider these compounds as peptide derivatives containing an oligonucleotide substituent in the side chain. Based on this principle, it was proposed to term compounds comprising amino acid (peptide) and nucleoside (nucleotide) residues as 'nucleoamino acids' and 'nucleopeptides'.[13] In the abbreviated form, the name of the oligonucleotide is indicated in parentheses immediately after the symbol designating the corresponding amino acid, *e.g.*, H-Ala-Ser(pATAT)-Ala-OH. This approach is often used for compilation of names of conjugates where the peptide components are linked to oligonucleotides through a phosphodiester bond formed by the OH group of the hydroxy amino acid residue and the terminal phosphate of the oligonucleotide.

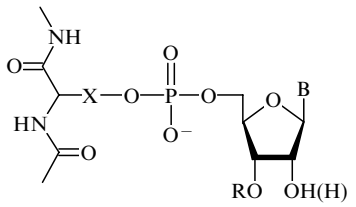
An alternative approach to the nomenclature of this class of mixed biopolymers was suggested by Z A Shabarova.[4] All compounds containing amino acid (peptide) and nucleotide (oligonucleotide) fragments were termed as peptide – nucleotide conjugates. The name of a concrete compound is made up of two components, *viz.*, the name of a nucleotide residue and the name of an amino acid (peptide). Between these names, the designation of the type of the linkage is placed in parentheses. Thus a compound in which the phenylalanine residue is linked to uridine 5′-phosphate by a phosphoramide bond will be termed as uridilyl-(5′→N)-phenylalanine. Such nomenclature has been extensively used to describe peptide – nucleotide conjugates with a phosphodiester or a phosphoramide bond between the fragments.

The main difficulties arise in the compilation of names for those conjugates where the oligonucleotide and peptide components are connected through a special linker possessing a complex structure. In this case, the conjugate actually consists of three components. The authors of the overwhelming majority of publications prefer to give more general names to such compounds. In foreign literature, it is the term 'peptide – oligonucleotide conjugates' ('hybrids') that has become especially popular.

II. Synthesis of peptide – oligonucleotide conjugates in solutions

1. Conjugates with phosphodiester bonds between the fragments

Studies of covalent complexes isolated from adenoviruses, polyoviruses and certain bacteriophages [2,3] have demonstrated that the protein component of these compounds is linked to the nucleic acid through a phosphodiester bond formed by the OH group of the hydroxy amino acid and the 5′-terminal phosphate group of the polynucleotide.

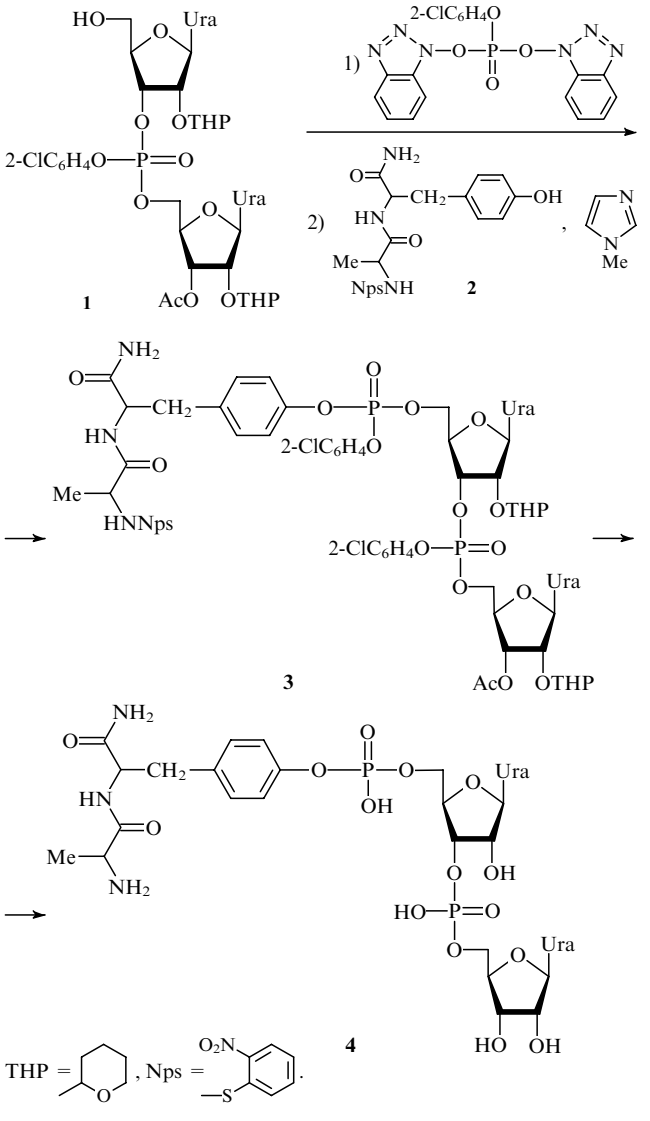


X = $-CH_2-$, $-CH_2CH_2-$, $-CH(CH_3)-$, $-CH_2C_6H_4-$;
R, a polynucleotide chain;
B, hereinafter, a heterocyclic base.

It has been found that topoisomerase I-induced transformations of DNA involve the formation of a DNA – protein structure in which the protein is covalently bound to the cleavage site of a double helix chain through a tyrosine residue.[14]

In order to simplify the analysis of complex NA – protein structures, it seems expedient to use model compounds, *e.g.*, peptide – oligonucleotide conjugates of the phosphodiester type. In this context, the main attention is given to the development of efficient procedures for the synthesis of these compounds. Thus van Boom *et al.*[15] have proposed to this end phosphorylation of the hydroxy group of a peptide by the 5′-phosphate group of an oligonucleotide. Partially protected dinucleoside phosphate **1** and the *N*-blocked dipeptide amide, Nps-Ala-Tyr-NH_2 (**2**) (Scheme 1), were used as the key compounds.[15] An alternative

Scheme 1



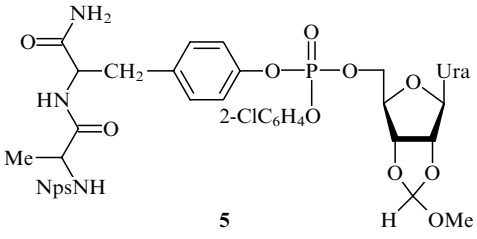
approach to the synthesis of peptide – oligonucleotide conjugates of the phosphodiester type is based on the reaction of an *O*-phosphorylated peptide and an oligonucleotide with a free 5′-hydroxy group.

In both cases, the investigators had to solve at least two problems, *viz*., to choose an optimum method for the activation of the phosphate residue and to search for adequate procedures for the protection of all functional groups of the peptide and the oligonucleotide which are not to be phosphorylated or involved in the condensation. It is of note that the removal of the protective group should be carried out under mild conditions to prevent side reactions.

2-Chlorophenyl bis(benzotriazolido) phosphate in pyridine was used for the phosphorylation and simultaneous activation of dinucleoside phosphate **1**. The dipeptide **2** was added to the activated derivative formed in the presence of *N*-methylimidazole to give a fully protected peptide – oligonucleotide conjugate **3** in 80% yield. The latter was isolated by column chromatography on silica gel. Of extreme importance is the fact that the condensation of the oligonucleotide and peptide components leads to the formation of a phosphotriester bond between them. In an alternative procedure for the synthesis of compound **3**, the hydroxy group of the tyrosine constituent of the peptide is phosphorylated by the bifunctional phosphorylating reagent after which dinucleoside phosphate **1** is added to the reaction mixture. The yield of the peptide – oligonucleotide conjugate **3** prepared by this method was 78%.

Deprotection of the functional groups in compound **3** giving the targed product **4** was carried out using the following reaction sequence.[15] First, both 2-chlorophenyl phosphate-protective groups were removed by treatment with the oximate ion. The main difficulty in this deprotection is connected with the presence of an additional aryloxy group in one of the phosphotriesters formed through the tyrosine side chain. Since in this case the pK_a values of the leaving groups are close to that of the remaining aryloxy group, the possibility of selective scission of the 2-chlorophenyl group under the action of *syn*-4-nitrobenzaldoxime and N^1,N^1,N^3,N^3-tetramethylguanidine was problematic.

In order to examine the possibility of selective removal of the 2-chlorophenyl protective group, the authors performed the synthesis of a model compound **5**.



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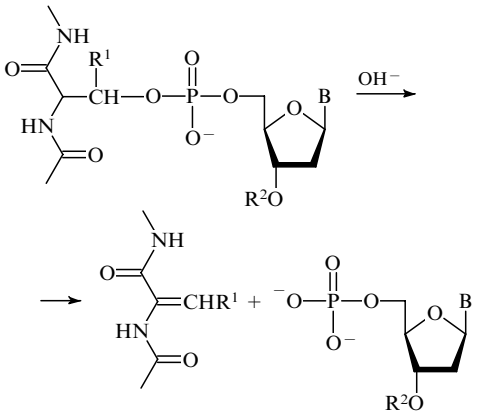
The reaction products formed upon its deprotection did not contain dipeptide **2**. The structure of H-Ala-Tyr(pU)NH_2 was confirmed by ^{31}P NMR spectroscopy. Thus, it was concluded that the 2-chlorophenyl phosphate-protective group could be selectively removed with *syn*-4-nitrobenzaldoxime and N^1,N^1,N^3,N^3-tetramethylguanidine.[15]

The removal of the 3′-*O*-acetyl group from compound **3** was effected by treatment with triethylamine in aqueous methanol. The 2-nitrophenylsulfenyl (Nps) and the 2′-*O*-tetrahydropyranyl (THP) groups were cleaved at room temperature by treatment with hydrogen chloride in methanol for 3 h and with aqueous HCl (pH 2) for 14 h.

The study by the same group of Dutch investigators[16] is considered to be a logical continuation of their previous work.[15] An attempt was undertaken to develop a reliable procedure for the preparation of peptide – oligonucleotide conjugates of the phosphodiester type with the DNA fragment linked to the peptide through a serine or a threonine residue. As a matter of fact, the problem in the synthesis of such peptide – oligonucleotide conjugates is associated with the high lability of the phosphodiester bond. This circumstance significantly complicates the deprotection and restricts the range of the reagents used.

The analysis of published data[3,4] prompts a conclusion that the stabilities of the phosphodiester bonds in oligonucleotidyl-(5′→O) – peptides depend on the nature of the hydroxy amino acid due to the difference in the mechanisms of cleavage of the phosphodiester bonds. If the tyrosine residue is involved, the bond is stable in moderately acidic and alkaline media, but is cleaved under strongly alkaline conditions in exactly the same way as in ordinary phosphoric acid diesters. If the phosphodiester bond involves a serine or a threonine residue, this undergoes β-elimination in alkaline media with the liberation of a phosphomonoester group, *i.e*., of an oligonucleotide containing 5′-terminal phosphate. In the case of serine, the peptide fragment formed as a result of this reaction contains a dehydroalanine residue (Scheme 2).

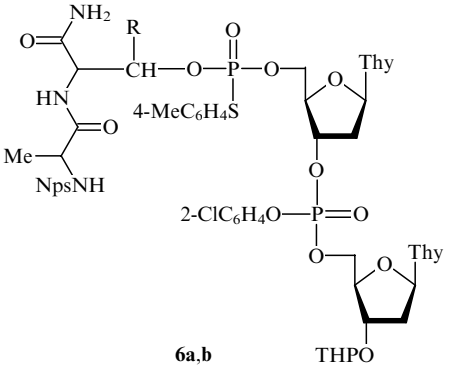
Scheme 2



R^1 = H, Me; R^2, polynucleotide chain.

The hydrolytic stabilities of serine and threonine derivatives are different,[3] the latter being more resistant to alkaline treatment.

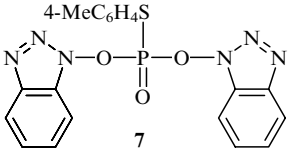
The conjugates **6a**,**b**



6a,**b**

R = H (**a**), Me (**b**)

were prepared[16] using a procedure similar to that described above. *S*-*p*-Tolyl bis(benzotriazolido) phosphothioate (**7**) was used as a phosphorylating reagent, since the *p*-tolylsulfenyl group (Mps) is split off under mild conditions.[16]



7

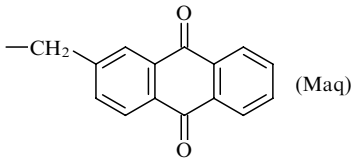
The yield of compound **6a** was 80%. Compound **6b** was prepared in a relatively low yield (50%), which can be attributed to low solubility of the dipeptide Nps-Ala-Thr-NH_2 in a dioxane – pyridine mixture.

Removal of the Mps group from the completely protected peptide–oligonucleotide conjugate **6a** was performed by treatment with an excess of silver acetate in aqueous pyridine. The splitting of the Mps group was completed in 48 h at room temperature. However, the deprotection of the internucleotide phosphate by the oximate ion led to the cleavage of the phosphodiester bond between the components of the hybrid molecule **6a** by the β-elimination mechanism (^{31}P NMR spectroscopic data). It is of note that the removal of the phosphate-protective groups in compound **6b** using the same reagents did not affect the phosphodiester bond. Tetrabutylammonium fluoride has proved to be a milder reagent for the splitting of the 2-chlorophenyl protective group. The removal of Nps and THP groups was performed with 0.01 M HCl (pH 2) (0–5 °C, 16 h).

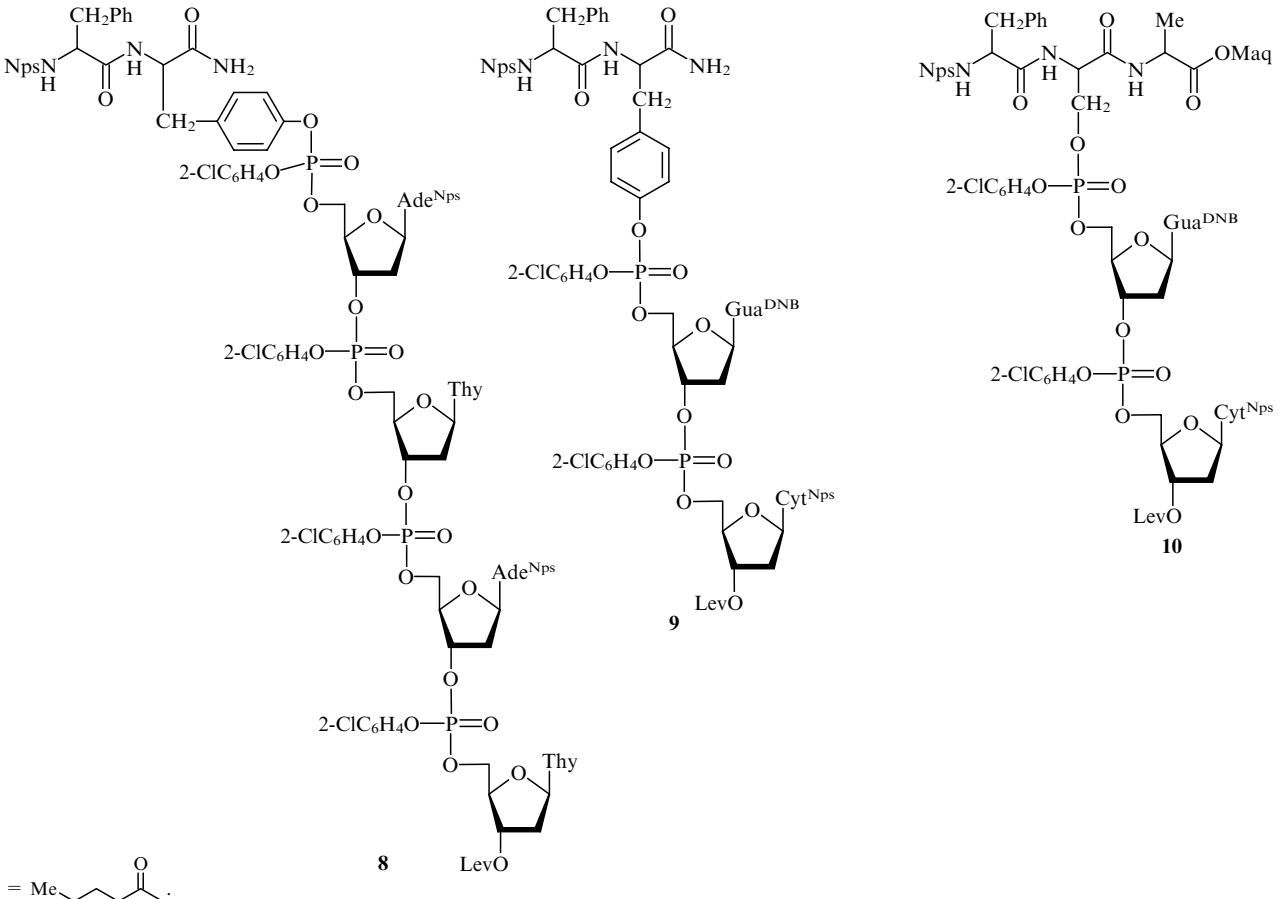
The stabilities of the peptide–oligonucleotide conjugates H-Ala-Ser(pTT)-NH_2 and H-Ala-Thr(pTT)-NH_2 in alkaline media have been studied.[16] Studies by ^{31}P NMR spectroscopy have demonstrated that H-Ala-Ser(pTT)-NH_2 was completely hydrolysed by 0.15 M NaOH (20 °C, 25 min) to form the dinucleotide pTT, whereas no complete hydrolysis of H-AlaThr(pTT)-NH_2 occurred after 9 h treatment with alkali under identical conditions.

In further studies, the same group of investigators used this approach to obtain conjugates with a heterooligonucleotide component.[17,18] The problem arising in such a synthesis relates to the necessity of protection of exocyclic amino groups of the heterocyclic bases. The authors had to abandon the use of acyl protective groups employed in routine oligonucleotide syntheses, because their removal by treatment with concentrated ammonia leads to the cleavage of the phosphodiester bond between the oligonucleotide and the peptide formed with the involvement of the hydroxy group of serine or threonine. This could result in the base-catalysed racemisation of amino acid residues. Therefore, the recourse to the protective groups commonly employed in peptide synthesis was made in the synthesis of peptide–oligonucleotide conjugates.

Van Boom *et al.*[15] have protected the NH_2 groups of adenine and cytosine with the Nps group. The latter is relatively easily introduced, is stable under conditions of phosphorylation and increases the stability of N-glycosidic bond of purine nucleosides in acid media. However, an attempt to synthesise N^2-nitrophenylsulfenyldeoxyguanosine failed.[18] Therefore, advantage was taken of *N,N*-di-n-butylformamidine protection (N=CHNBu$^n_2$, DNB) of the exocyclic NH_2 group of guanine, which withstands column chromatography on silica gel and can be removed by mild treatment with hydrazine. It should be noted that it was exclusively amides of *N*-protected peptides that have been used as starting components for the synthesis of peptide–oligonucleotide conjugates in early studies,[15,16] while later Dutch investigators used peptides with terminal carboxy groups protected as allyl (All) [17] or anthraquinon-2-ylmethyl (Maq) esters.[18]

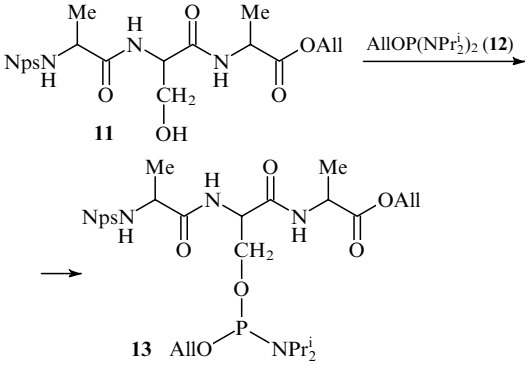


The approaches to the synthesis of the peptide–oligonucleotide conjugates H-Phe-Tyr(pATAT)-NH_2, H-Phe-Tyr(pGC)-NH_2 and H-Phe-Ser(pGC)-Ala-OH (see Refs 17, 18) are, on the whole, similar to those proposed in earlier studies,[15,16] but the procedures used for the deprotection of the intermediate fully protected peptide–oligonucleotide conjugates **8**–**10** have been modified: Nps-protection was cleaved with 2-mercaptopyridine, while 3′-levulinoyl (Lev) and DNB protections were removed by hydrazine hydrate.

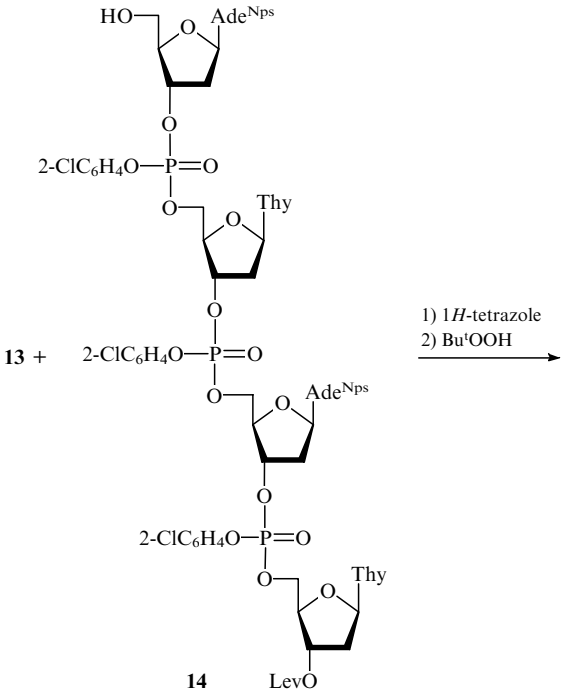


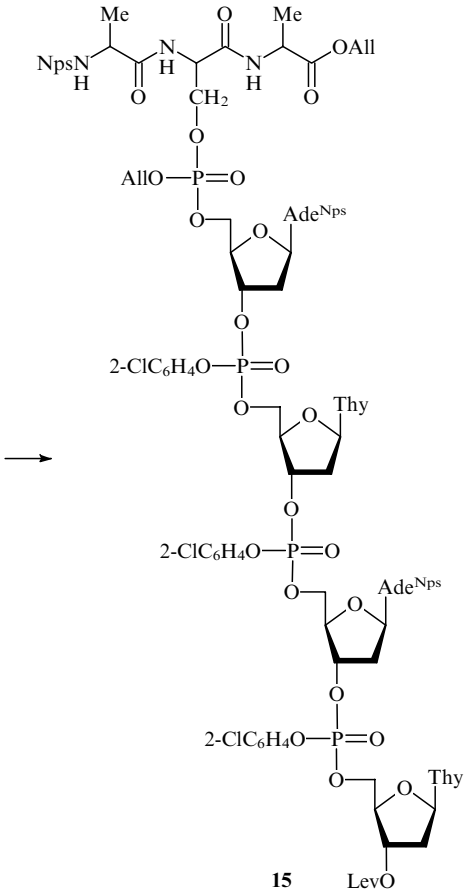
The phosphotriester method has initially been used to prepare H-Ala-Ser(pATAT)-Ala-OAll.[17] The yield of the fully blocked peptide – oligonucleotide conjugate was 64%. The removal of protective groups was carried out by the same method as that used for compound **8**. An alternative approach consisting of phosphorylation of the tripeptide Nps-Ala-Ser-Ala-OAll by 2-chlorophenyl bis(benzotriazolido) phosphate and subsequent reaction of the activated tripeptide derivative with the partially protected tetramer ATAT was inefficient. The phosphorylation of the hydroxy group of serine led to the formation of the undesired symmetrical product.

These difficulties were successfully overcome through the use of the phosphoramidite method.[17]



The tripeptide **11** was first treated with equimolar amounts of 1*H*-tetrazole and the phosphitylating reagent **12** in acetonitrile after which equimolar amounts of the tetramer **14** and 1*H*-tetrazole were added to the phosphite **13** formed. The duration of the reaction was 18 h. The phosphite unit in the resulting conjugate was oxidised to the phosphate by treatment with *tert*-butyl hydroperoxide.





The notable advantage of this method is the use of the allyl protective group in the phosphotriester bridge between the tripeptide and the oligonucleotide in the completely protected peptide – oligonucleotide conjugate **15**, which is easily removed with aqueous pyridine. The only disadvantage of the phosphotriester and phosphite versions of H-Ala-Ser(pATAT)-Ala-OAll synthesis is that the carboxy group of the C-terminal amino acid could not be recovered by transition metal complexes.

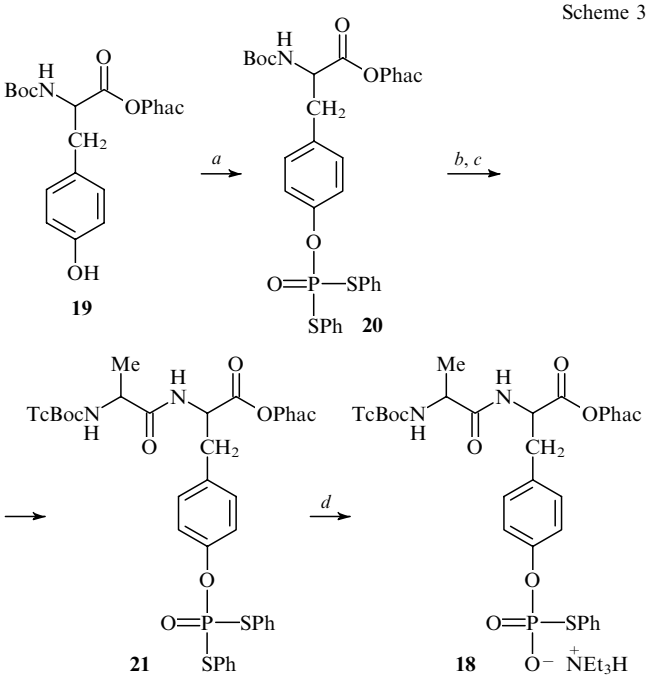
Of special interest are the studies by Japanese investigators[19,20] who have developed an original procedure for the preparation of peptide – oligonucleotide conjugates of the phosphodiester type, *viz.*, H-Ala-Tyr(pUU)-OH (**16**) and H-Ala-Ser(pTT)-Phe-OH (**17**) based on the chemistry of phosphothioates.

Synthesis of the phosphorylated dipeptide component **18** was carried out using the Boc-strategy (Scheme 3).[19]

In the first step, the partially protected tyrosine derivative **19** was phosphorylated by *S*,*S*-diphenyl phosphodithioate (PSS) in the presence of isoduroldisulfonyl chloride (DDS). N-protected alanine was added to the phosphorylated tyrosine derivative **20** after deprotection of the amino group. *N*,*N'*-Dicyclohexylcarbodiimide and 1-hydroxybenzotriazole were used as the condensation reagent in the formation of the peptide bond. Selective splitting of one phenylthio group in the derivative **21** was achieved by treatment with triethylammonium hypophosphate in pyridine.[19]

The phosphorylated tripeptide **22** was prepared in a similar way.[20] In this case, the hydroxy group of serine was phosphorylated by *S*,*S*-bis(*p*-methoxyphenyl) phosphodithioate (MPSS).

Scheme 3



Boc = $-C(=O)-OBu^t$, Phac = $-CH_2-C(=O)-Ph$, TcBoc = $-C(=O)-O-C(Me)_2-CCl_3$;

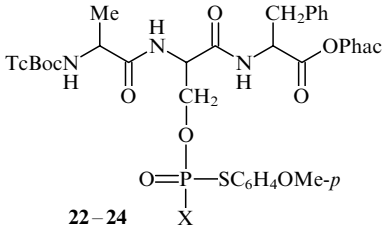
(*a*) $^-O-P(=O)(SPh)-SPh$ (PSS), 2,4,6-trimethylbenzene-1,3-bis(sulfonyl chloride) (DDS), 1*H*-tetrazole;

(*b*) 4 M HCl – dioxane; (*c*) TcBoc-Ala-OH,

cyclo-$C_6H_{11}N{=}C{=}NC_6H_{11}$-cyclo (DCC), 1-hydroxybenzotriazole (HOBT);

(*d*) $Et_3\overset{+}{N}H$ $H_2PO_2^-$, C_5H_5N.

However, treatment of compound **22** with a solution of triethylammonium hypophosphate in pyridine yielded MPSS rather than compound **23**.[20] Presumably, this was due to β-elimination of phosphodithioate. Therefore, selective removal of one *p*-methoxyphenylthio group was achieved by milder treatment with bis(tributyltin) oxide which afforded compound **24**.



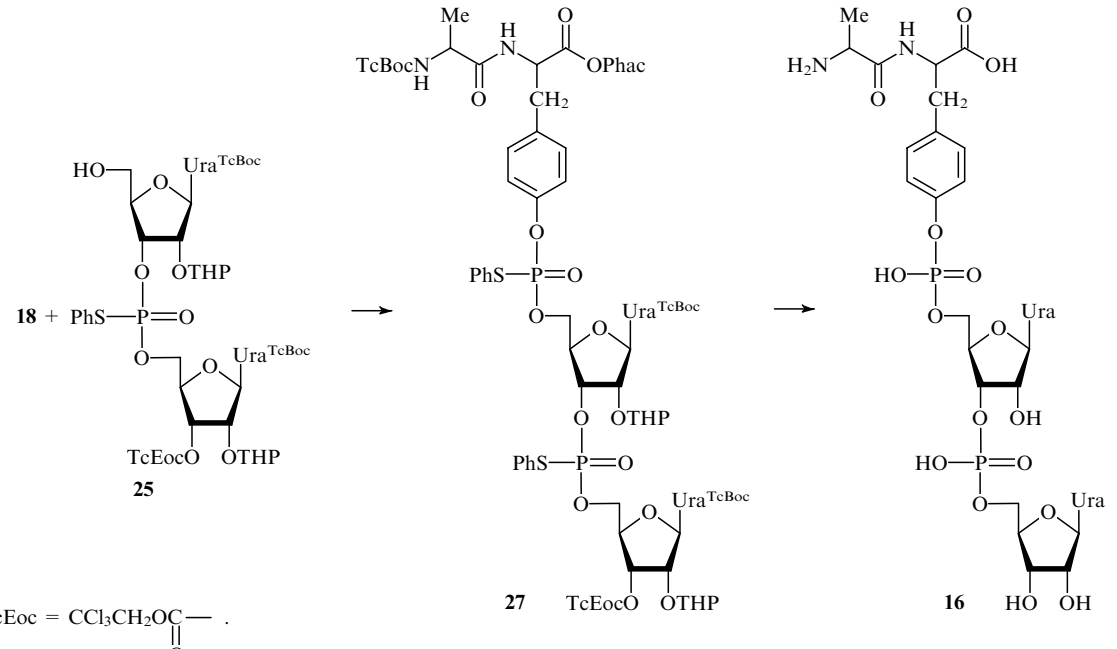
X = SC_6H_4OMe-*p* (**22**), O^- $Et_3\overset{+}{N}H$ (**23**), $OSnBu^n_3$ (**24**).

The syntheses of partly protected dinucleotide components **25** and **26** (Schemes 4 and 5) made use of PSS and DDS as bifunctional condensation reagents. The uracil and thymine N^3-TcBoc protective group can be removed by reduction with zinc in acetylacetone under neutral conditions.

In the synthesis of the target peptide – oligonucleotide conjugate **16**, compounds **18** and **25** were introduced into the reaction with DDS and 3-nitro-1,2,4-triazole (Scheme 4). The fully protected peptide – oligonucleotide conjugate **27** was obtained in 73% yield.[19]

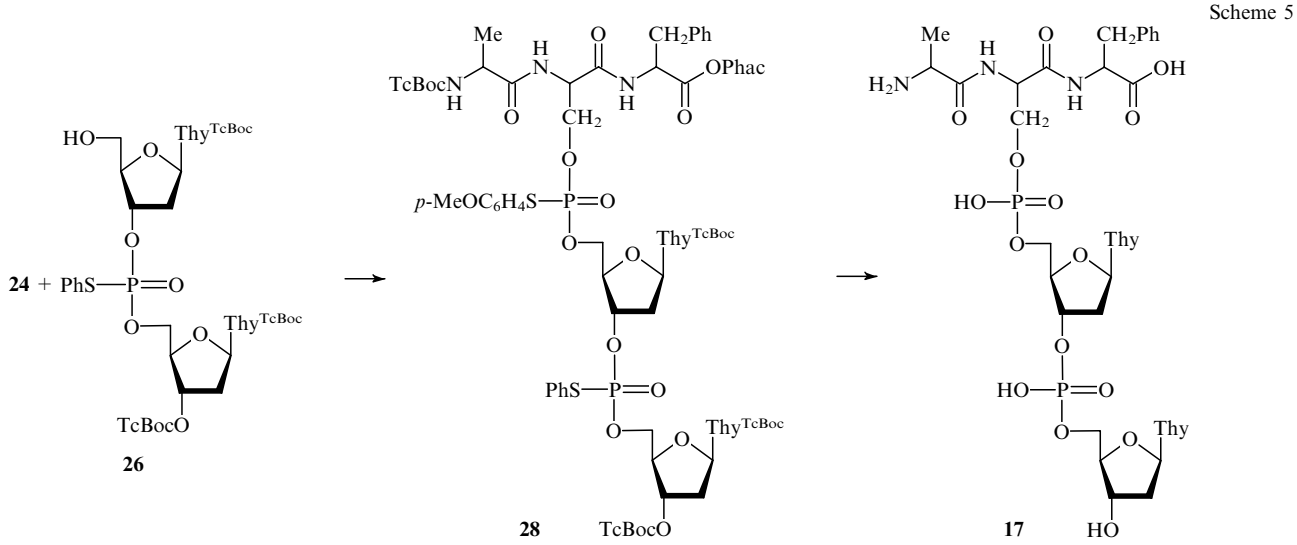
The use of the same reagents in the synthesis of the peptide – oligonucleotide conjugate **17** (Scheme 5) results in the formation of both the target compound **17** and the tripeptide containing a dehydroalanine residue. Therefore, the reaction was carried out in the presence of *N*-methylimidazole and an excess of triisopropylbenzenesulfonyl chloride (TPSCl).[20] Successive deprotection of compounds **27** and **28** afforded the conjugates **16** and **17**, respectively. The phosphate groups were deprotected with an excess of $(Bu^n_3Sn)_2O$. The TcBoc, Phac and TcEoc groups were cleaved by reduction with zinc in acetylacetone. The 2′-*O*-tetrahydropyranyl groups were removed from compound **27** using a standard procedure (0.01 M HCl, pH 2).

Scheme 4



TcEoc = $CCl_3CH_2OC(=O)-$.

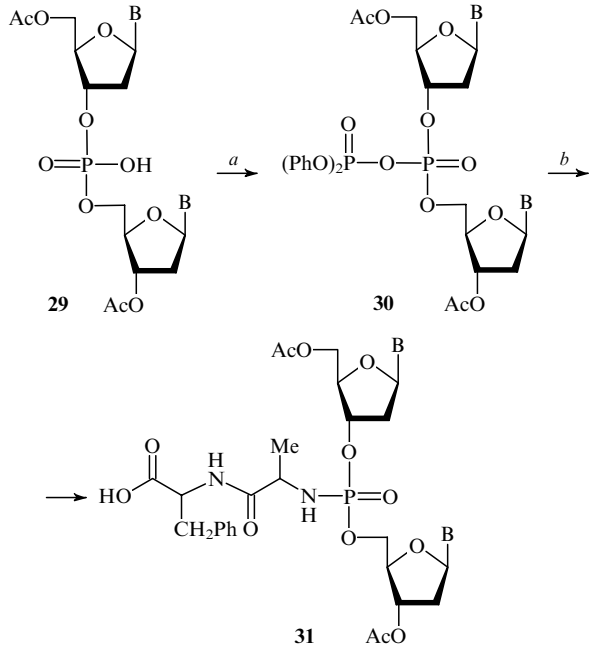
Scheme 5



2. Conjugates with phosphoramide bonds between the fragments

From the historical point of view, mixed biopolymers with the phosphoramide bonds between the oligonucleotide and the peptide fragments were the first to be synthesised. The possibility of modification of the internucleotide phosphate group was demonstrated by Prokof'ev, Shabarova *et al.*[5,21] in the example of dinucleoside phosphates (Scheme 6).

Scheme 6



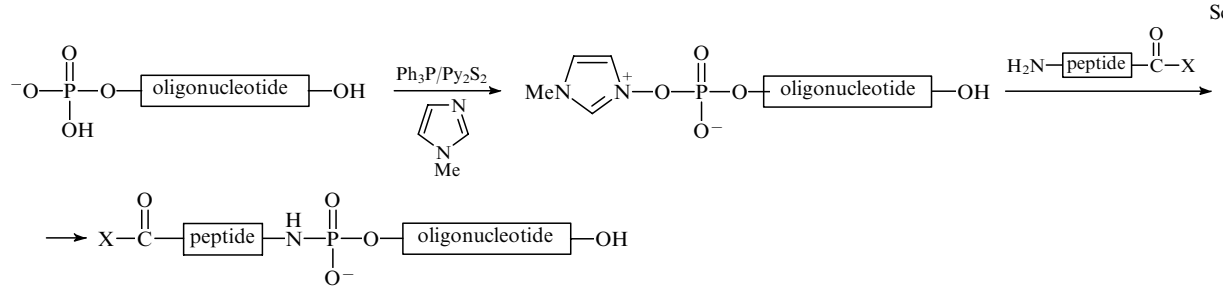
(*a*) $(PhO)_2P(O)Cl$; (*b*) H-Ala-Phe-OH.

Treatment of dinucleoside phosphate **29** containing protected hydroxy groups with diphenylphosphochloridate yielded the corresponding non-symmetrical pyrophosphate **30**. The latter represented an active phosphorylating reagent. Reaction of pyrophosphate **30** with the dipeptide H-Ala-Phe-OH is accompanied by nucleophilic substitution at the internucleotide phosphorus atom; the role of the leaving group is played by the diphenyl phosphate anion. The advantages of this method are as follows: (*i*) the synthesis of pyrophosphate does not affect heterocyclic base residues; (*ii*) pyrophosphate can be introduced into a reaction with the nucleophile without preliminary isolation. The yield of the target product **31** was 75%. However, this method [5] gave satisfactory results only in the case of deoxyribo derivatives; in the presence of a protected 2′-hydroxy group, the degree of conversion was only 10%.

An alternative approach to the synthesis of peptide – oligonucleotide conjugates of the phosphoramide type was developed by a group of Novosibirsk investigators.[22–25] The α-amino group of the peptide reacts with preactivated 3′- or 5′-terminal phosphate of the oligonucleotide. The advantage of this approach is the possibility to use non-protected fragments of nucleic acids. This, in turn, is due to the fact that the phosphate residue is selectively activated with a mixture of triphenylphosphine and 2,2′-dipyridyl disulfide in the presence of the nucleophilic catalyst *N*-methylimidazole (Scheme 7). Under these conditions, the internucleotide phosphate groups and the reactive centres of heterocyclic bases are not involved. The mechanism of this activation of phosphoric acid monoesters is considered in detail in the review by Zarytova.[26]

In the study,[22,23] the 5′-terminal phosphate group was activated in a mixture of dimethylformamide and dimethyl sulfoxide. The oligonucleotides were solubilised in organic solvents as cetyltrimethylammonium (cetavlon) salts. The activation of the phosphate residues of the oligonucleotide gives rise to *N*-methylimidazolide which reacts with the amines with pK_a values in a very

Scheme 7

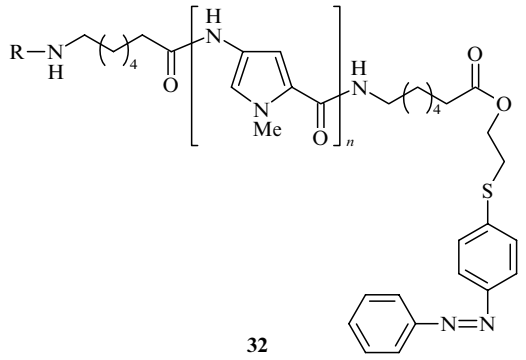


X = OMe, NH_2.

broad range; with an increase in pK_a, the rate of the phosphoramide bond formation increases. The guanidine group of arginine in the peptides H-Pro-Arg-Val-OMe and H-(Leu-Arg)$_n$-Gly-NH$_2$ (n = 2–4) is basic. In order to prevent the reaction of the latter with the activated phosphate group of the oligonucleotide, the peptides were introduced into the reaction in the form of trifluoroacetate salts. Under these conditions, the guanidine group remains protonated throughout the reaction and the role of the main nucleophilic centre after selective deprotonation by triethylamine is played by the α-amino group of the peptide.

The presence of a P—N bond in the peptide–oligonucleotide conjugates formed was confirmed by ^{31}P NMR spectroscopy and acid hydrolysis under conditions of cleavage of the phosphoramide bond (0.1 M HCl, 18 h). The target peptide–oligonucleotide conjugates were isolated by reversed-phase HPLC in 70%–85% yields. It was noted [23] that the addition of the peptide to the oligonucleotide increases the hydrophobicity of the latter. In the reversed-phase HPLC, the retention time of the reaction product increases in comparison with that of the original oligonucleotide.

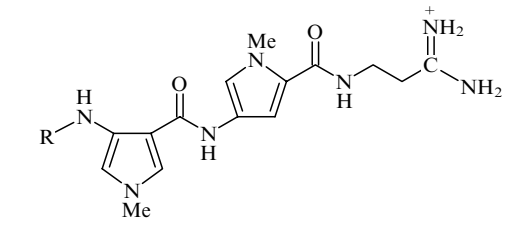
The method described above was used for the conjugation of a synthetic analogue of the peptide antibiotic netropsin with oligothymidylic and oligodeoxyadenylic acids.[24] In this case, the peptide was attached to the 3′-end of the oligonucleotide. The yields of the conjugates **32** were 30%–50%.

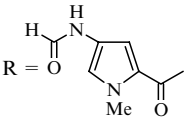
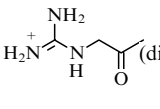


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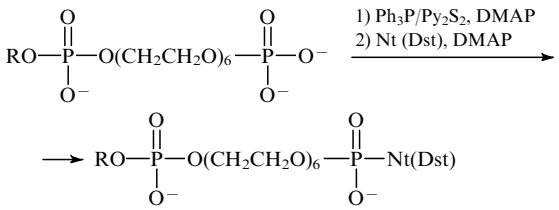
n = 2–5; R, fragment of the oligonucleotide chain.

A somewhat different approach was proposed for the synthesis of covalent complexes of non-modified oligonucleotides and their thiophosphate analogues with netropsin (Nt) or yet another peptide antibiotic, *viz.*, distamycin (Dst).[25]



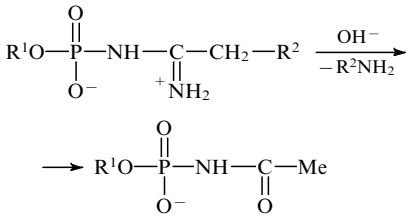
R =  (netropsin, Nt),  (distamycin, Dst).

Automated phosphoramidite oligodeoxyribonucleotide synthesis was used to prepare oligonucleotides bearing, at their 3′- or 5′-ends, hexaethylene glycol linkers with phosphate groups. This was followed by the activation of the terminal phosphate groups by redox reagents of the types Ph_3P and Py_2S_2 in the presence of 4-dimethylaminopyridine (DMAP), after which a peptide antibiotic (Nt or Dst) was added to the activated oligonucleotide together with an excess of DMAP. The latter is thought [25] to be necessary for neutralisation of the positive charges of the amidine and guanidine groups.



R, fragment of the oligonucleotide chain.

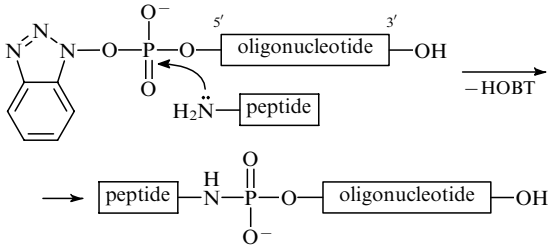
Presumably,[25] a covalent P—N bond formed between the antibiotic and the NA fragment involves the amidine group of the antibiotic. Indirect evidence can be derived from the fact that the rates of hydrolysis of oligonucleotide netropsin and acetamidine derivatives under the action of concentrated aqueous ammonia are identical. Apparently, the hydrolysis is accompanied by deamination:



R^1, fragment of the oligonucleotide chain, R^2, residue of the antibiotic.

A group of investigators representing the Moscow research team led by Z A Shabarova [27] have developed a method for the carbodiimide-induced activation of terminal phosphate groups of oligonucleotides by *N*-hydroxybenzotriazole (HOBT). HOBT esters are rather stable in aqueous media and can effectively phosphorylate nucleophilic reagents with the formation of covalent bonds (Scheme 8). Amino acids and peptides can be used as nucleophilic reagents.

Scheme 8



This was applied to the synthesis of the Gly-Gly-(5′→N)-(pTCTAG) conjugate [28] and to the synthesis of oligodeoxyribonucleotides with the antibiotic polymixin B1, which represents a polycationic peptide attached to the 5′-terminal phosphate group.[29] The yields of the product reached 80%. An undecanucleotide conjugate has been designed and successfully prepared with 3′-terminal phosphate attached to the antibiotic daunomycin and the 5′-terminal phosphate attached to polymixin B1, the latter serving to facilitate the penetration of the oligonucleotide through the cell membrane. This conjugate was isolated by polyacrylamide gel electrophoresis (PAGE); its total yield was 20%.

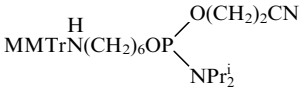
The extension of these methods [27–29] to RNA derivatives was a logical continuation of the studies.[30] Conditions for effective synthesis of HOBT esters of 5′-oligoribonucleotides were selected. The formation of a specific complex of this duplex with the peptide Tat was demonstrated in the example of a synthetic RNA complex possessing a binding site for the protein Tat HIV-1. The covalent coupling of the activated RNA duplex to the peptide proceeded with 20%–30% efficiency and did not require any additional activation. Thus, the peptide Tat–TAR RNA complex is an example of effective covalent coupling of a protein to RNA within a specific complex.

Synthesis of a conjugate of a triplex-forming oligonucleotide with a functionally important peptide has recently been described. Such a conjugate is a convenient tool for the study of modulation of gene expression.[31,32]

3. Conjugates with amide bonds between the fragments

The studies[33–40] are devoted to the synthesis of peptide – oligonucleotide conjugates with the amide bond between the fragments formed as a result of the reaction of the activated α-carboxy group of the peptide with the aliphatic NH_2 group incorporated into the oligonucleotide.

The aliphatic amino group is usually attached to the 5′- or the 3′-end of the oligonucleotide through a polymethylene spacer in the course of automated phosphoramidite synthesis. Commercially available 6-aminohexyl 2-cyanoethyl *N*,*N*-diisopropylphosphoramidite, the amino group of which is protected by the 4-monomethoxytrityl (MMTr) group, a convenient reagent for the preparation of DNA fragments with 5′-terminal NH_2 groups.

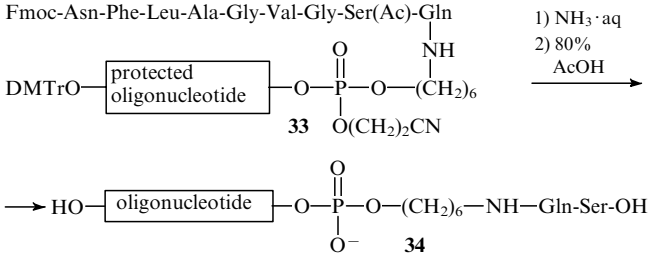


The synthesis of oligonucleotides containing a 3′-aminoalkyl group is carried out with the use of a special UV-cleavable polymeric support with a photolabile anchor containing a terminal aminohexanol group (Scheme 9). The attachment of the first nucleotide is performed in an automated DNA synthesiser, whereas the splitting of the oligonucleotide with the 3′-terminal amino group incorporated is effected by UV irradiation.[41] The main advantage of this method is that the presence of a photolabile anchor group facilitates the synthesis of protected oligonucleotides with only the aliphatic amino group free.

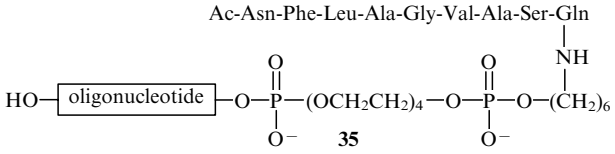
Such a partially protected oligonucleotide was introduced into a condensation with one of the two *N*-blocked tripeptides, *viz*., Fmoc-Gly-Gly-Gly-OH (Fmoc is 9-fluorenylmethoxycarbonyl), or Fmoc-Gly-Gly-His-OH (see Scheme 9). The activation of the carboxy groups in the tripeptides was carried out by a $Ph_3P - Py_2S_2$ system in the presence of DMAP. The reaction was carried out in dimethylformamide at room temperature because of the possibility of cleavage of the Fmoc group and concomitant oligomerisation of the tripeptide under more drastic conditions. The yield of the peptide – oligonucleotide conjugates after deprotection and isolation by ion-exchange HPLC or PAAG electrophoresis was 89% – 99%.

The condensation of short peptides and oligonucleotides using redox reagents was shown[42] to be accompanied by epimerisation of the C-terminal amino acid residue. Model experiments showed that the degree of epimerisation reached 30% under these conditions (^{1}H NMR spectroscopic data).

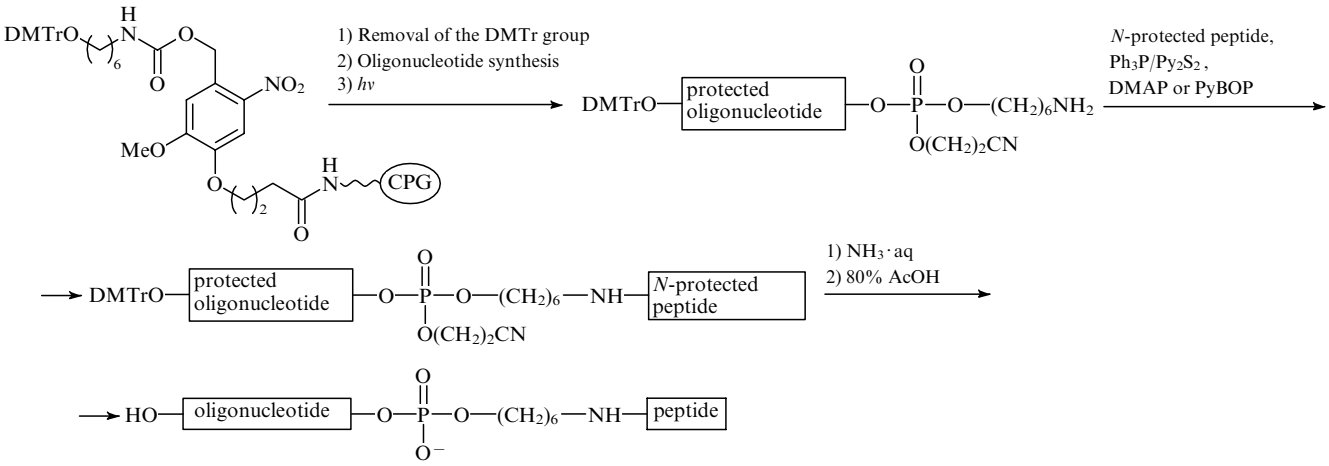
An attempt has been made[34] to prepare peptide – oligonucleotide conjugates with long peptide chains. In order to activate the terminal carboxy group of the peptide, the authors used (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP).[34] However, mass spectrometric analysis performed after deprotection of the peptide – oligonucleotide **33** formed and isolation of the target product revealed that its peptide component contains only two amino acid residues (compound **34**).



It is suggested[34] that the cleavage of the peptide component at the amide bond between the serine and glycine residues was due to N→O acyl migration. Apparently, the free OH group of serine liberated upon treatment with ammonia attacks the amide bond between the serine and glycine residues, which is followed by the scission of the peptide chain due to hydrolysis of the ester bonds. The use of milder procedures for the removal of protective groups (0.4 M NaOH in methanol, 17 h) did not give any positive results either. This problem could be overcome by replacing the glycine residue by the alanine residue in order to create steric hindrances in the site of possible nucleophilic attack. Some other changes necessary for further formation of a stable helical complex between the peptide – oligonucleotide conjugate and the complementary NA target were also effected, *viz*., the α-amino group of the peptide was protected by the acetyl group and an additional tetraethylene glycol bridge was introduced between the 3′-terminal nucleotide and the alkylamine in order to separate the peptide fragment from the DNA chain. In this case, the yield of the conjugate **35** was 72%.

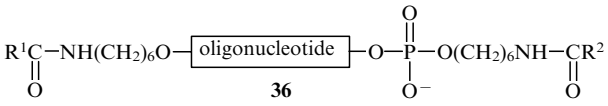


Scheme 9



GPG, controlled pore glass;
DMTr, 4,4′-dimethoxytrityl protective group; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate.

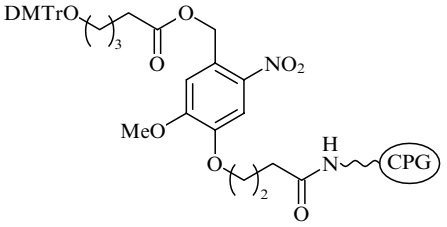
The peptide–oligonucleotide 3′,5′-bisconjugates **36** were successfully obtained using the polymeric support mentioned above in the first step. The peptide–oligonucleotide 3′-conjugates were synthesised according to Scheme 9 after which the 5′-terminal HO group was made to react with commercially available 6-aminohexyl 2-cyanoethyl *N*,*N*′-diisopropylphosphoramidite to which another peptide was then attached.[35]



36

R^1,R^2, peptide residues.

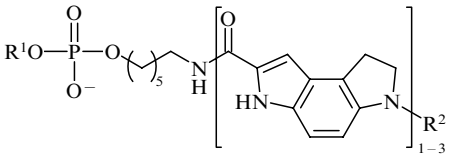
The use of protected oligonucleotides with 3′-terminal carboxy groups for the synthesis of peptide–oligonucleotide conjugates was a natural continuation of these studies.[36] A UV-cleavable polymeric support was also employed.



The covalent bond between the 3′-end of the oligonucleotide and the N-end of the peptide is easily formed even in the case of spatial hindrance created by the N-terminal amino acid. Excellent results were obtained in the coupling of tetrapeptides; the conjugates were obtained in 89%–95% yields.

Special mention should also be made of a synthesis of a series of peptide conjugates by coupling them to non-protected oligonucleotides T_6 and T_{10} containing 5′-terminal NH_2 groups using PyBOP as the condensation reagent.[37]

Synthesis of covalent oligonucleotide complexes with a synthetic analogue of the peptide antibiotic CC-1065 has been described.[38]

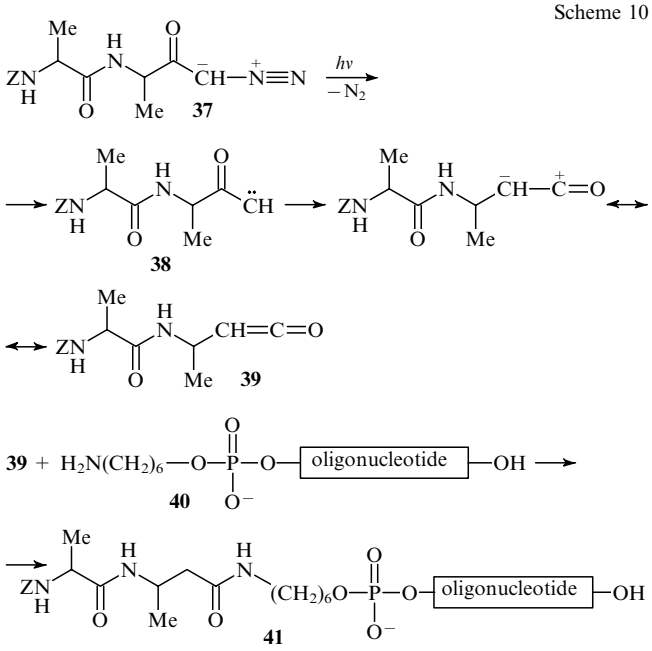


R^1, oligonucleotide chain, R^2 = $CONH_2$, Boc.

This method includes condensation of 2,3,5,6-tetrafluorophenyl ester of a peptide and a deprotected oligonucleotide bearing a 5′- or 3′-terminal amino group. The reaction is carried out in dimethyl sulfoxide. No detailed description of the synthesis of the DNA fragments containing a 5′- or 3′-terminal aminohexyl residue is provided. The oligonucleotides were introduced into the reaction as cetavlon salts. The yields of the condensation products were 60%–90% (HPLC data). It is noted that precipitation of the reaction products by the addition of a 2% solution of $LiClO_4$ in acetone and their further isolation by reversed-phase HPLC decreases the total yield to 15%–50%.

An interesting approach to the synthesis of peptide–oligonucleotide conjugates containing an amide bond was proposed by Swiss investigators.[39] Their method is based on the Wolff rearrangement. Diazo ketone **37** was used as a peptide component, and an oligothymidylate, as an oligonucleotide. The reaction was carried out in dimethylformamide. The diazo ketone **37** is decomposed upon UV irradiation with abstraction of the nitrogen molecule to give carbene **38** which undergoes the Wolff rearrangement to be further converted into ketene **39** (Scheme 10). The latter reacts with oligothymidylate **40** containing a 5′-terminal amino group. The yield of the peptide–oligonucleotide conjugate

Scheme 10

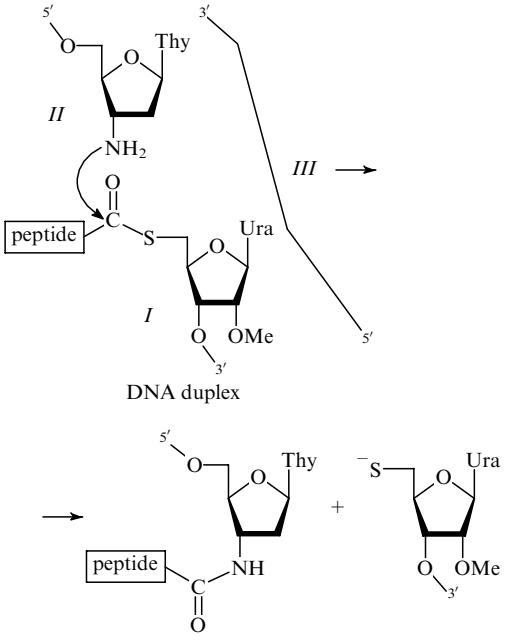


Z = $PhCH_2OCO$.

41 is 35%. The attempts to introduce diazo ketone **37** into the reaction with heteropolymeric DNA molecules were unsuccessful. The authors attribute this phenomenon to the poor solubilities of the heteropolymers in dimethylformamide. The presence in the reaction mixture of water molecules competing with oligonucleotides for the reaction with ketenes is an alternative explanation for the low yields.

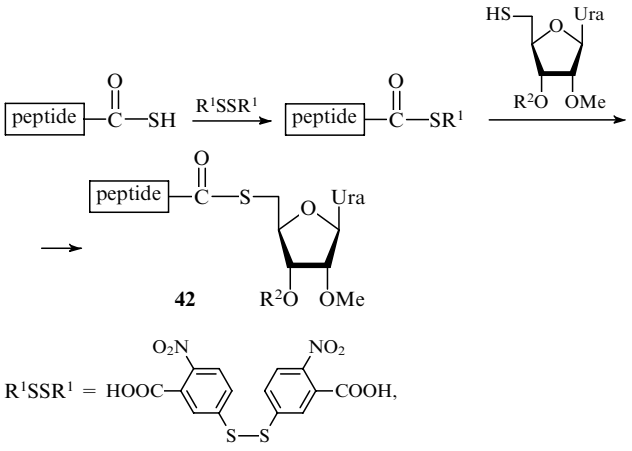
The approaches discussed above to the synthesis of oligonucleotide peptides largely entail the use of reactions occurring in organic solvents. An original procedure for the formation of covalent bonds between peptides and oligonucleotides in aqueous solutions based on the chemical ligation method has been developed.[40] This consists in approaching the reacting groups in the terminal fragments of the oligonucleotides *I* and *II* as a result of complementary interactions of these oligonucleotides with the complementary adjacent sites of the oligonucleotide *III* (Scheme 11).

Scheme 11



The peptides used in the study [40] were prepared in the form of carboxythionic acids by solid-phase synthesis on a polymeric support. Treatment of these peptides with Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)] gave the carboxythionic acid *S*-ester. The latter reacted with an oligonucleotide containing a 2′-*O*-methyl-5′-deoxy-5′-thiouridine to give the oligonucleotide peptide **42** with the thioester bond (Scheme 12). The reaction was carried out for 30 min at 25 °C in a buffer solution (pH 8.0) in the presence of spermidine. The product formed **42** is stable at room temperature for 24 h, but is easily hydrolysed at 37 °C over the same period.[40]

Scheme 12



R^1SSR^1 = HOOC– –

R^2, oligonucleotide chain.

The condensation of the peptide linked to a single-stranded fragment of the oligonucleotide *I* with the oligonucleotide *II* containing 3′-terminal 3′-amino-3′-deoxythymidine within the DNA duplex is the key step in the synthesis of peptide – oligonucleotide conjugates possessing amide bonds (see Scheme 11). A DNA duplex represents a highly ordered system; in its cleavage site, the terminal 3′-amino group of one oligonucleotide and the thioester group of the other peptide are located in close proximity to one another and are spatially oriented in a definite manner owing to the interactions stabilising the double helix. As a consequence, the chemical reaction is characterised by high selectivity and high rate, which are unattainable for this type of conversion occurring outside the helical complex. Under optimum conditions (2 – 4 mmol of spermidine, pH 8, 37 °C), the condensation was completed in 15 – 30 h; the peptide – oligonucleotide conjugates were obtained in good yields (60% – 85%). Owing to the weakly alkaline medium, the 3′-amino group remained non-protonated, while the presence of spermidine in the reaction mixture favoured additional stabilisation of the DNA duplex.

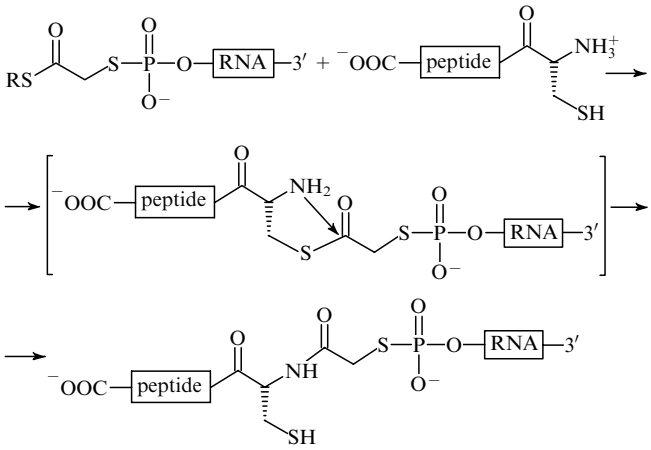
This approach allows simultaneous synthesis of several peptide – oligonucleotide conjugates possessing different structures. This is possible when an oligonucleotide with a 3′-amino group is involved in the simultaneous formation of two different DNA duplexes as has been demonstrated in the study under discussion.

Peptides of various lengths underwent condensation with oligonucleotides. However, reactions with short peptides encountered serious difficulties because of possible formation of cyclic structures. This is due to the fact that the α-amino group of the peptide, along with the 3′-amino group of the DNA fragment, can attack the carbonyl carbon atom resulting in the formation of a cyclic peptide. Moreover, yet another prerequisite for efficient condensation is that the C-terminal amino acid residue of the peptide did not contain a branched side chain, *i.e.*, its C-end should be devoid of isoleucine or threonine residues.

The use of a non-modified oligonucleotide with a 3′-terminal hydroxy group in the condensation is also noteworthy.[40] Here, the formation of an ester bond between the peptide and the DNA fragment occurs; the yields of peptide – oligonucleotide conjugates with ester bonds are lower than those with amide bonds. In contrast to the amide bond, the ester bond is easily hydrolysed in alkaline media (0.01 M NaOH) at 25 °C for 1 h.

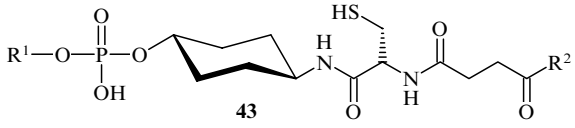
A more recent report [43] describes selective non-matrix chemical ligation of modified RNA containing the 5′-*S*-thioester group and polypeptides with N-terminal cysteine residues which affords peptide – 5′-RNA conjugates (Scheme 13).

Scheme 13



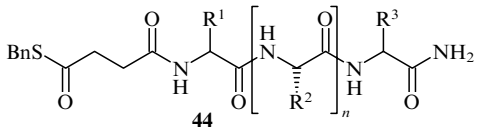
This reaction included transthioesterification and subsequent spontaneous intramolecular rearrangement resulting in the formation of a peptide bond. The authors observed high selectivity of ligation (50% yield of the conjugate of 102-membered RNA with a 13-membered peptide) at low (micromolar) concentrations of the reactants. This reaction proceeds under non-denaturing conditions favouring the preparation of NA – protein conjugates with simultaneous preservation of the native structure of the protein. The synthesis of peptides carrying N-terminal cysteine residues does not present a problem. The 5′-terminal phosphothioate residue can be easily introduced into a ribo- or deoxyribonucleic acid either chemically or enzymatically.

The term 'native ligation' (by analogy with condensation of two unprotected peptide fragments, one of which contains a C-terminal *S*-carboxythioate group and the other one contains an N-terminal cysteine, which is routinely used in peptide chemistry [44]) was used [45,46] to describe a new procedure for the preparation of peptide – oligonucleotide conjugates **43** (in the absence of a template).

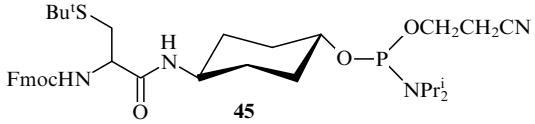


R^1, fragment of an oligonucleotide chain,
R^2, fragment of a peptide chain.

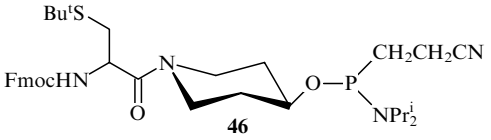
This method is based on the reaction of the peptide **44** modified by the introduction of the *S*-thioester group in its N-end with an oligonucleotide containing a 5′-terminal cysteine residue which is introduced using compound **45**.



R^1,R^2,R^3, side chains of amino acids.



S-Benzyl thiosuccinate **44** was obtained in the last step of a standard automated peptide synthesis (Fmoc strategy). The *O-trans*-4-(*N*$^{\alpha}$-Fmoc-*S-tert*-butyl-L-cysteinyl)aminocyclohexanol phosphoramidite **45** was attached to the 5′-end of the oligonucleotide in the course of a standard solid-phase phosphoramidite synthesis. The preparation of the phosphoramidite synthon **46** based on 4-hydroxypiperidine has also been described.[46]



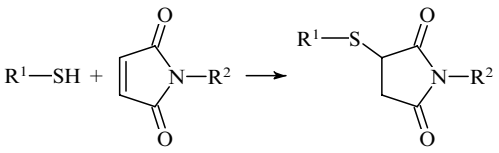
46

The peptide **44** and the oligonucleotide modified by compound **45** (or **46**) after deprotection and detachment from the resin were condensed without additional purification in water–organic solvents to which tris(2-carboxyethyl)phosphine (in order to remove the *S-tert*-butyl group *in situ*) and thiophenol (in order to increase the efficiency of thiol exchange) were added.

Some reactions between peptides and oligonucleotides of various sequences and lengths have been described and the ways to increase the efficiency of their synthesis have been considered.[45] The peptide–oligonucleotide conjugate **43** was the main product in all cases; sometimes, its yield reached 75%. The side reaction, *i.e.*, intramolecular cyclisation involving the succinyl fragment of the peptide, took place only with glycine at the N-end. No doubt, further experiments are necessary in order to optimise the composition of the solvent, reaction conditions (particularly, for long-chain peptides with a secondary structure). However, even now it is quite clear that the method in question [45] can be used for the preparation of oligonucleotide conjugates with a very broad range of peptides with small restrictions for amino acid sequences. This approach has obvious advantages over the method considered above for the synthesis of peptide–RNA conjugates,[43] since it allows the use of peptides with thioesters at their C- and N-ends and a 5′-cysteinyl-substituted oligonucleotide prepared by a standard phosphoramidite procedure. The method [43] allows the use of exclusively N-terminal cysteine-containing peptides and RNA modified with 5′-phosphothioate.

4. Conjugates with sulfide bonds between the fragments

The optimum variant of peptide–oligonucleotide synthesis seems to be the one in which the reactive group of the oligonucleotide reacts selectively with the reactive group of the peptide, whereas other functional groups of both macromolecules are not involved. For example, the conjugation can be effected by the addition of thiols to maleimides.

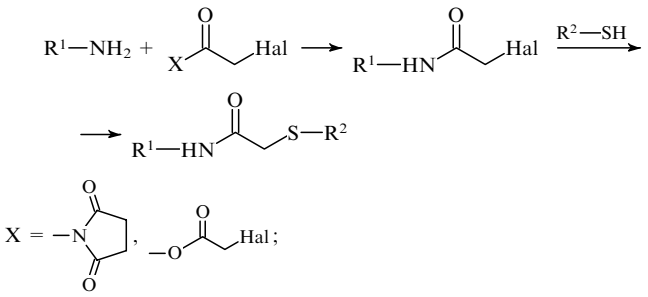


R^1,R^2, an oligonucleotide or a peptide chain fragments.

The sulfhydryl or the maleimide active groups are first introduced into the oligonucleotide or the peptide using various reagents. It should be noted, however, that the role of a reaction centre can be played by the HS group of the cysteine residue initially present in the peptide. It is preferable, as a rule, that the sulfhydryl group is localised in the oligonucleotide, whereas the maleimide group, in the peptide.[47–58] An alternative variant [59–63] is that the HS group of the N- or C-terminal cysteine residue reacts with the maleimide group incorporated into the oligonucleotide chain.

In both cases, the reaction is carried out in aqueous or water–organic media (pH 6.0–7.2) at room temperature. The peptide is almost always taken in an excess (from 4 to 15 equiv.); in only one study, the ratio between the two components of the reaction mixture was 1 : 1.[48] The reaction time varies from 1 to 20 h. The yields of the conjugates vary within a sufficiently broad range, *viz.*, from 30% to 90%.

The formation of a thioether bond between the oligonucleotide and the peptide can also take place as a result of a nucleophilic attack of the HS group at the carbon atom of the halogenoacetyl derivative. For this purpose, the halogenoacetic acid residue is incorporated into oligonucleotides or peptides.[64–72]

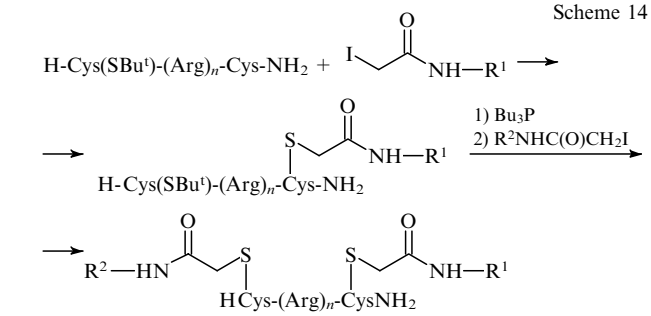


R^1,R^2, an oligonucleotide or a peptide chain fragments.

As a rule, the oligonucleotide containing an iodoacetyl group reacts with an excess of the cysteine-containing peptide in neutral media at room temperature.[64–67] The reaction time is 12–20 h. The yields of the conjugates usually vary from 55% to 98%. Reed *et al.*[68] performed the synthesis in a weakly alkaline medium (pH 8.3). The authors cite interesting results concerning the dependence of the reaction rate on the net charge of the peptide. Thus the reaction of a modified oligonucleotide with a peptide containing one arginine residue and four lysine residues (the net charge is +5) is complete within a few minutes. In the case of the other two peptides with the net charges of +1 and zero, the reaction time is 3 and 20 h, respectively. The authors presume that the increase in the condensation rate is caused by electrostatic interactions of the negatively charged phosphates in the DNA molecule with the positively charged side groups of lysine and arginine of peptides.

The synthesis of peptide–oligonucleotide conjugates in which two DNA fragments are linked through a peptide bridge has been described (Scheme 14) [64] with H-Cys(SBu^t)-(Arg)$_n$-Cys-NH$_2$ (n = 3, 5, 7) as a bridge.

Scheme 14



R^1,R^2, fragments of oligonucleotide chains; n = 3, 5, 7.

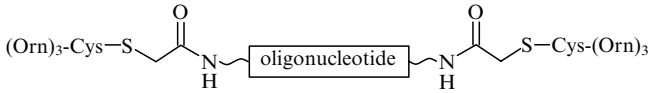
Initially, the free HS-group of the C-terminal cysteine residue reacted with the iodoacetyl group localised at the 5′-end of one oligonucleotide. In order to prevent the removal of the *S-tert*-butyl group, it was necessary to carry out the reaction in a neutral medium. After termination and chromatographic isolation of the peptide–oligonucleotide conjugate, the second thiol group was deprotected by treatment with a large excess of tributylphosphine at room temperature for 4 h. Taking into consideration low solubility of tributylphosphine in water, the removal of the protective group was carried out in a biphasic water–dichloromethane system.

The authors presume that tributylphosphine has a salient advantage over dithiothreitol which is widely used for the reductive cleavage of the S—S bond. On termination of the reaction, the excess of dithiothreitol should be removed, whereas in reactions with tertiary phosphine the separation of the organic phase from

the aqueous phase is sometimes sufficient. It was found that complete cleavage of the *S-tert*-butyl group should be carried out first, and only after this has been done, another oligonucleotide containing an iodoacetyl group at its 3′-end is to be added to the reaction mixture. Otherwise, the removal of the *S*-protective group occurs at a slow rate, since iodoacetic acid and its amide can form quaternary phosphonium salts with Bu_3P.

The reaction of the HS group of the N-terminal cysteine residue of the peptide – oligonucleotide conjugate with the electrophilic centre of the second oligonucleotide proceeded in a weakly alkaline medium, which made it possible to increase the yield of the target product. It should be noted also that all the reactions were carried out in a lithium chloride solution.[64] The reason lies in poor solubility of the peptide – oligonucleotide conjugate in water due to the interactions of the phosphate groups of DNA with positively charged guanidine groups of arginine, which are attenuated by the presence of lithium chloride. At the same time, the oligonucleotide pairs containing a peptide bridge are readily soluble in water because they contain an excessive (with respect to the guanidine groups) amount of negatively charged phosphates.

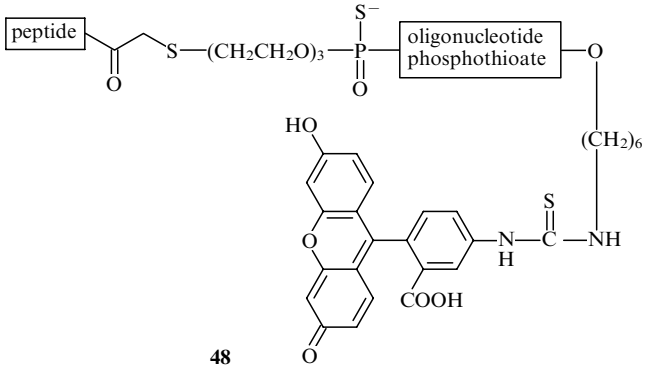
This approach was also used to prepare the conjugate **47** the two peptide fragments in which were attached to the 3′- and 5′-ends of the oligonucleotide.



47

In the studies,[69–72] the bromoacetic acid residue was introduced into the peptide fragment prior to the condensation with an oligonucleotide containing an HS group. The bromoacetyl derivative of the peptide was taken in excess (from 5 to 25 equiv.). The reaction was carried out at room temperature for 5 – 20 h. The yield of the target product was 70% – 100%. The effect of the medium acidity (pH 7.0, 7.5, 7.9 and 8.5) on the completeness of the reaction and the dependence of the reaction rate on temperature have been studied.[69] It was found that the peptide – oligonucleotide conjugate was formed in quantitative yield in less than 3 h at 40 °C and pH 7.0 and 7.5.

The publication[72] devoted to the synthesis of the complex fluorescently labelled peptide – oligonucleotide conjugate **48** provides an illustrative example of a successful application of the above-described procedure to the synthesis of peptide – oligonucleotide conjugates. This was preceded by the synthesis of 19 – 25-membered oligodeoxyribonucleotide phosphothioates containing a 5′-terminal protected thiol group and an amino group at the end of the linker attached to its 3′-end. This bisfunctionalised oligonucleotide was introduced into the reaction with a fluorescein isothiocyanate (FITC) fluorescent label at the 3′-amino group; the thio group was then deprotected and reacted with the *N*-bromoacetyl derivative of the peptide.

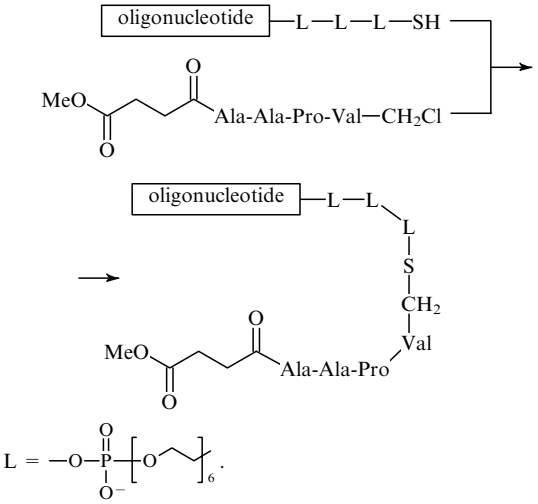


48

Special emphasis is laid on the possibility of performing large-scale synthesis of the conjugate **48**. The success of this synthesis depended on the development of a new procedure for deprotection of oligonucleotides in which the oligomers were treated with a mixture of dipyridyl disulfide and concentrated ammonia in the presence of phenol and methanol. This deprotecting mixture enables splitting of the oligonucleotide from the polymeric support, liberation of the 3′-terminal amino group, deprotection of the phosphate and amino groups of heterocyclic bases and the conversion of the 5′-terminal thioacetyl protective group into the pyridyl disulfide group. The presence of phenol in the deprotecting mixture makes it possible to avoid the side reaction associated with a large-scale synthesis. Usually, the deprotection of internucleotide phosphates affords an electrophile, *viz*., acrylonitrile ($CH_2=CHCN$), which reacts with the nucleophilic sulfur of the (oligonucleotide – linker – SH) system to give the undesirable product oligonucleotide – linker – SCH_2CH_2CN.

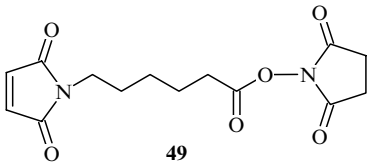
The synthesis of peptide – oligonucleotide conjugates based on the reaction of peptidyl chloromethyl ketone with an oligonculeotide containing a 3′-terminal thio group has been described (Scheme 15).[73]

Scheme 15

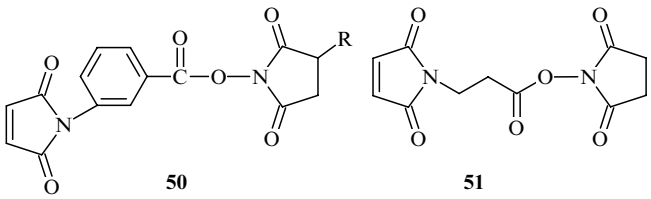


The conjugation of the oligonucleotide with the peptide was carried out in a water – organic solution (pH 7.5) at room temperature for 12 – 16 h. The yield of the target product was 60%.

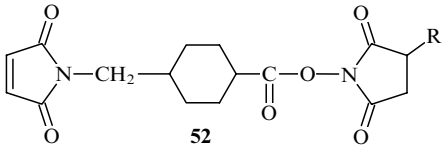
Activated esters of ε-maleimidohexanoic (**49**),[47,48] 3-maleimidobenzoic (**50**),[51,53,54,59,60] β-maleimidopropionic (**51**)[55,56] and 4-maleimidomethylcyclohexanecarboxylic (**52**)[57,61–63] acids were used as heterobifunctional reagents for the incorporation of the maleimide group into peptides and oligonucleotides.



49



50 **51**



52

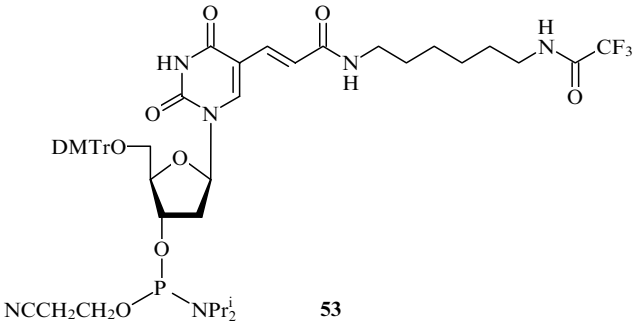
R = H, SO_3Na.

In the study,[48] the α-amino group of the peptide was acylated by the reagent **49** in water at pH 7.0 for the incorporation of the maleimide group into the peptide. This reaction was also carried out in methanol in the presence of triethylamine.[47] In the majority of cases, the peptide was prepared by solid-phase synthesis; after deprotection, the free α-amino group was acylated by one of the above-mentioned bifunctional reagents in the presence of 1-hydroxybenzotriazole.[51, 53–56]

Low storage stability of maleimide peptide derivatives containing lysine residues in aqueous media was noted.[51] A complex mixture of reaction products is formed after a period of 3–4 days as a result of the reaction of the ε-amino groups of lysine residues with the maleimide group. To prevent side reactions, it is proposed to introduce the modified peptide into the reaction immediately after its detachment from the polymeric support.

It was recommended to incorporate the bromoacetic acid residue into peptides by performing the acylation of the peptide ε-group by bromoacetic acid anhydride in the course of the solid-phase synthesis.[69–71]

The synthesis of maleimide derivatives of oligonucleotides includes two steps, *viz*., the automated oligonucleotide synthesis followed by modification of the oligomeric chain formed by 6-(4-monomethoxytrityl)aminohexyl 2-cyanoethyl *N*,*N*-diisopropylphosphoramidite.[59–62] The aliphatic amino group was introduced into the predetermined position of the DNA fragment using a commercially available 3′-phosphoramidite derivative of the modified nucleoside **53**.[63]



53

After splitting of the oligonucleotide from the polymeric support and removal of protective groups, the aliphatic amino group reacted with one of the heterobifunctional reagents **49**–**52** containing a maleimide group (pH 7–8). The average yields of the oligonucleotide maleimide derivatives are 60%–70%.

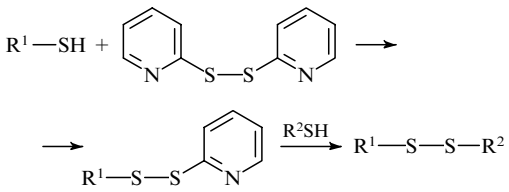
The methods for the preparation of oligonucleotides containing iodoacetic acid are similar. The amino group attached to the 3′- or 5′-end of the oligonucleotide through a hexamethylene bridge is acylated by *N*-hydroxysuccinimide ester[64–66] or iodoacetic anhydride in a water–organic mixture.[68]

5. Conjugates with disulfide bonds between the fragments

To this point, we have dealt with various procedures for the synthesis of conjugates, the oligonucleotide and peptide components of which were linked by stable covalent bonds. However, the development of techniques for the synthesis of peptide–oligonucleotide conjugates with easily cleavable bonds between the fragments is a no less important task. Such a bond can be represented by an S—S bond, which can be formed and cleaved under conditions acceptable for various biological processes.

The synthesis of conjugates with disulfide bonds between their peptide and oligonucleotide fragments can be carried out using three main approaches. First, this is in the oxidation of the HS groups of the oligonucleotide and the peptide with atmospheric oxygen with the formation of a disulfide bridge. In this case, the peptide is taken in an excess, the yield of the conjugate is 60%.[61] However, according to Azhayev *et al.*[74] the yields of peptide–oligonucleotide conjugates prepared by this method are extremely low (0.4%–23%).

The other two approaches consist of the activation of the HS group of the oligonucleotide or the peptide with 2,2′-dipyridyl disulfide.[61, 74–81]

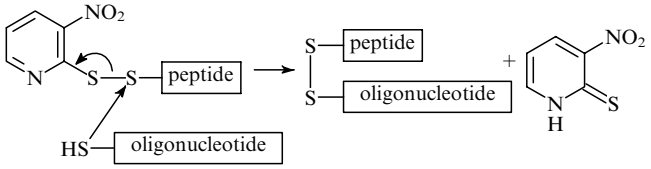


R^1,R^2, oligonucleotide or peptide chain fragments.

The reaction is carried out under mild conditions at room temperature.

Unprotected peptides and oligonucleotides can form non-covalent complexes due to electrostatic interactions of the negatively charged phosphates of DNA with positively charged side groups of the peptides. Such complexes are precipitated from solutions. Having regard to this circumstance, potassium chloride and acetonitrile, which neutralise molecular charges and facilitate their dissolution, are added deliberately to the reaction mixture.[76] It is also recommended to carry out the synthesis of the conjugates in 90% formamide.[74]

The introduction of the *S*-(3-nitro-2-pyridylsulfenyl)cysteine residue (Cys^{Npys}), which is Boc-protected at the α-amino group, is a distinctive feature of a conjugation method.[51, 69] It is noteworthy that the Npys-protective group is resistant to acid treatment. After cleavage from the polymeric support and removal of protective groups (with the exception of the Npys group), the peptide formed in the solid-phase synthesis is introduced into the reaction with a DNA fragment containing an HS-group under mild conditions. The reaction of the HS group of the oligonucleotide with the Npys-protected thiol group of the cysteine of the polypeptide yields a conjugate with the S—S bond between the peptide and the oligonucleotide fragments.



It should be noted that the HS group can be introduced into oligonucleotides in several ways. First, it can be attached to the 3′-(see Refs 54 and 56) or 5′-end (see Refs 51, 53, 55) of the DNA fragment using linkers of various lengths. Second, the synthesis of a protected nucleoside 3′-phosphoramidite containing a thiol group can be followed by its introduction into any predetermined position of the oligomeric chain by the automated oligonucleotide synthesis.

Earlier it was mentioned that it is cysteine-containing peptides that are usually coupled to oligonucleotides. However, some methods permit the introduction of HS groups into peptides devoid of the cysteine residues. Thus β-mercaptopropionic acid was attached to the N-terminal amino acid residue in the course of solid-phase peptide synthesis.[74]

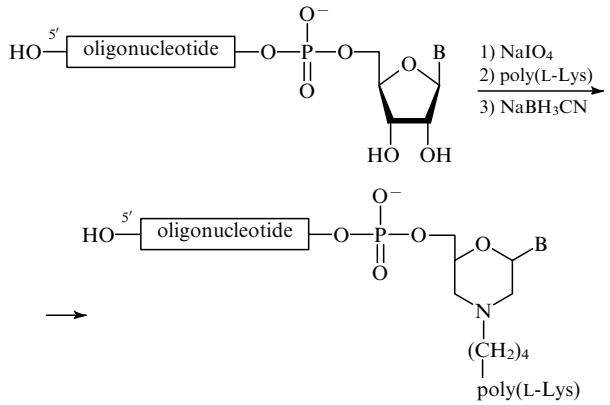
6. Synthesis of conjugates using a carbonyl derivative of one of the fragments

Syntheses of conjugates considered below are based on the reaction of carbonyl compounds with nucleophiles. The indisputable advantage of the carbonyl groups is that they do not require additional activation and their reactions proceed with high selectivities.

Dialdehyde derivatives of nucleic acids were extensively used in previous studies, *e.g.*, in the structural elucidation of the monomeric components of RNA and DNA. The methods for the introduction of aldehyde groups into carbohydrate fragments

of nucleic acids and the properties of the thus modified derivatives have recently been reviewed.[82,83]

Lebleu *et al.*[84–89] have performed a successful synthesis of peptide – oligonucleotide conjugates based on dialdehyde oligonucleotide derivatives. After periodate oxidation, the oligonucleotide with a 3′-terminal ribo fragment was subjected to condensation with poly(L-lysine) followed by reduction by sodium cyanoborohydride. The linkage between the peptide and the oligonucleotide fragments was effected through the morpholine ring formed.

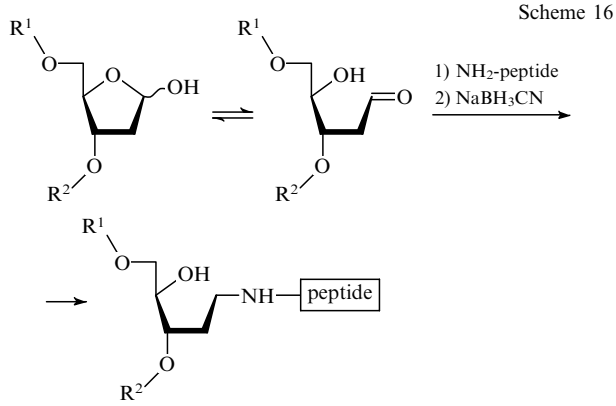


A $(2'\rightarrow5')(A)_n$-polyribonucleotide prepared by enzymatic synthesis was used as an oligonucleotide with a 3′-terminal ribo fragment;[84] in the case of oligodeoxyribonucleotide, this was introduced with the help of T4 RNA ligase;[85] the ribo fragment was the first to be attached to a polymeric support and the oligonucleotide was prepared by the automated synthesis.[87]

The early works of Lebleu *et al.*[84–87] reported the antisense properties of conjugates of dialdehyde oligonucleotide derivatives with peptides and their possible applications in the study of various biological systems. The results of testing of the complexes for the ability to inhibit replication of HIV-1 were published later (the oligonucleotide fragment of the complex was found to be complementary to the translation initiation centre of the Tat-protein).[88,89] Some sequence-specific antiviral effects of these compounds were observed.

In the study,[90] the electrophilic centres of oligonucleotides were generated enzymatically: the uracil residues were cut off from the oligonucleotide chain using uracil – DNA glycosylase to obtain the apurine/apyrimidine fragments. This was followed by modification of the aldehyde-containing oligonucleotides by various ligands carrying an amino group. In particular, the attachment of tripeptides to such oligonucleotides has been described (Scheme 16).

Scheme 16



R^1,R^2, oligonucleotide chain fragments.

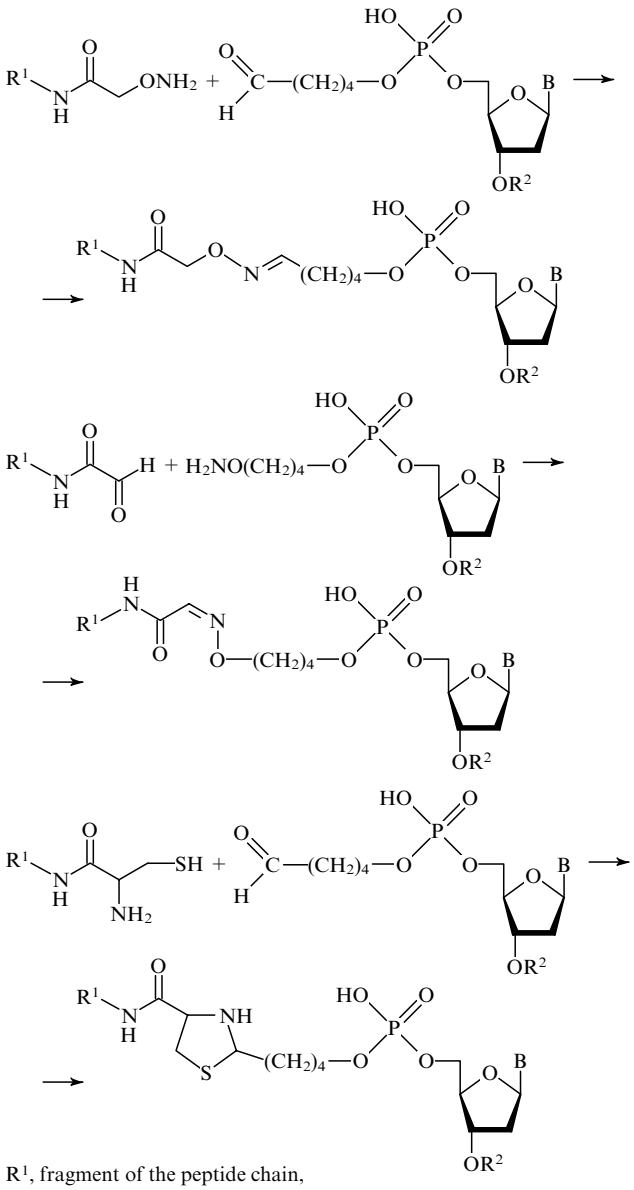
A highly efficient selective procedure for oligonucleotide synthesis involving the formation of oximes and thiazolidines has recently been proposed.[91] In their search for an 'ideal' conjugation procedure, *i.e.*, the simplest one-step reaction between a free peptide and an oligonucleotide under physiological conditions, the authors made their choice in favour of two novel types of binding groups, *viz.*, the oxime and the thiazolidine groups.

The synthesis of conjugates involving the formation of an oxime (reaction of an aldehyde with an aminooxy derivative) and thiazolidine (reaction of the aldehyde with 1,2-aminothiol) is shown in Scheme 17.

The aldehyde or the aminooxy group were incorporated into the oligonucleotide fragment at the 5′-end, while the peptide contained the corresponding complementary group, *viz.*, the aminooxy- or the 1,2-aminothiol groups for the reaction with the aldehyde-containing oligonucleotide or the α-*N*-glyoxyloyl group for the reaction with the aminooxy oligonucleotide derivative.

5′-Modified oligonucleotides were prepared by the standard automated synthesis using the corresponding modified 3′-phosphoramidite derivatives in the final step.[91] In turn, the N-terminal position or the side chain of lysine in the peptides was functionalised by introducing the aldehyde or the aminooxy groups by means of oxidative cleavage of the serine residue or using *N*-Boc-*O*-(carboxymethyl)hydroxylamine, respectively. The thiazolidine ring between the peptide and the oligonucleotide is formed in

Scheme 17



R^1, fragment of the peptide chain,
R^2, fragment of the oligonucleotide chain.

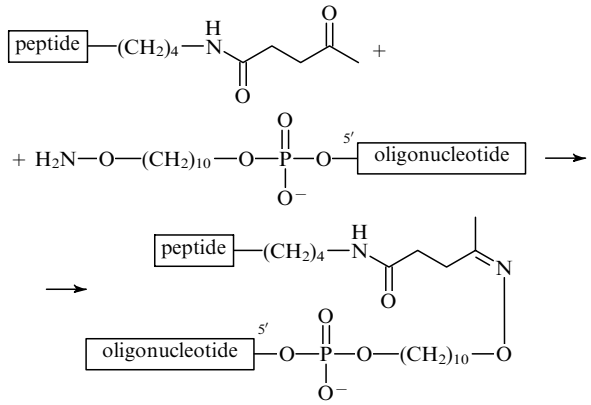
those cases where the original peptide is modified by attachment of cysteine at the N-end or at the side chain of lysine.

Oxime can also be formed in the DNA duplex. In this case, the 5′-aldehyde-containing oligonucleotide is hybridised with the complementary DNA chain and the DNA duplex formed is introduced into the reaction with a peptide carrying the aminooxy group. This strategy opens up fresh opportunities for post-synthetic modifications of single- and double-stranded DNA.

The formation of an oxime bond between the oligonucleotide and the peptide fragments occurs at a high rate and with high selectivity. However, oligonucleotides possessing aminooxy groups are seldom introduced into reactions with peptides carrying aldehyde groups, since such oligonucleotides easily react with any carbonyl groups and are therefore not very convenient. Those peptide–oligonucleotides in which the oligonucleotide and the peptide fragments are linked through the thiazolidine ring are less stable than those containing an oxime bond.

Later, the same authors [92] reported the synthesis of conjugates using oligonucleotides with 3′-terminal carbonyl groups. The 3′-terminal aldehyde group was generated by mild periodate oxidation of a 1,2-aminoalcohol attached to the 3′-end of the oligonucleotide in the automated synthesis on a standard commercially available polymer. The oligonucleotide thus prepared reacted with a peptide containing an aminooxy group similarly to the 5′-aldehyde-containing oligonucleotide.[91]

Simultaneously, another group of investigators [93] published a paper devoted to the synthesis of peptide–oligonucleotide conjugates from oligonucleotides containing 5′-terminal aminooxy groups and peptides containing N-terminal lysine groups with the side chains modified by the reaction with levulinic anhydride, *i.e.*, peptides with N-terminal keto groups. In this case, the binding of the oligonucleotide and the peptide fragments occurred due to formation of the oxime bond.



The preparation of conjugates involving the formation of oximes has been described by Dey and Sheppard.[94] The keto group was introduced into the C(5)-position of the oligonucleotide uracil through a spacer.

III. Solid-phase synthesis of peptide–oligonucleotide conjugates

The performance of chemical reactions on insoluble polymeric supports is one of the main techniques in modern organic synthesis. The idea underlying this method is virtually versatile and applicable to a wide variety of biopolymers and their analogues. In the past few decades, the solid-phase method has become a routine procedure in the synthesis of peptides and oligonucleotides; therefore, investigators give considerable attention to the development of strategies and tactics of chemical synthesis of peptide–oligonucleotide conjugates which is partly or completely performed on a solid phase.

This strategy is based on the construction of amino acid and nucleotide sequences which includes either stepwise elongation of peptide and oligonucleotide chains on the same solid support or condensation of the two preformed fragments. The second route implies the attachment of a preformed peptide to an oligonucleotide synthesised on a solid phase and immobilised on a polymeric support.

In development of the strategy of chemical synthesis of peptide–oligonucleotide conjugates, special emphasis is laid on the choice of optimum methods for the formation of amide and phosphodiester bonds and a search for the most adequate combinations of protective groups designed for peptides and nucleic acid fragments and conditions for the deprotection (the latter should be selected with due regard to the stability of the target peptide–oligonucleotide conjugates). The choice of method for the formation of a bond between the peptide and the oligonucleotide fragments is also of great importance.

1. Stepwise synthesis of peptide and nucleotide fragments on a single solid support

The synthesis of oligonucleotides and peptides includes a series of repeated operations involving structurally similar monomeric components. The sequential synthesis of the oligonucleotide and the peptide fragments of the hybrid molecule on a single solid support can raise serious problems connected with the impossibility, in principle, of combining some standard procedures inherent in the solid-phase oligonucleotide and peptide syntheses. Therefore, meticulous selection of methods and optimisation of experimental protocols are necessary in order to ensure acceptable yields of target products and to avoid the formation of by-products.

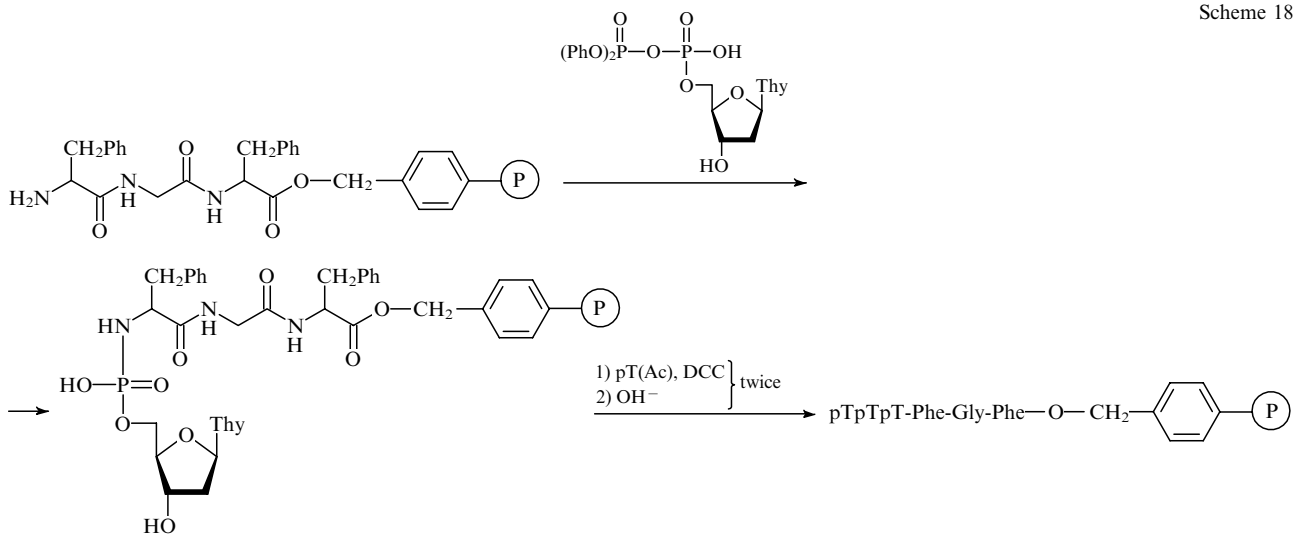
In 1972, M A Prokof'ev, Z A Shabarova *et al.* published a paper of fundamental importance [6] in which they gave the first description of a solid-phase synthesis of peptide–oligonucleotide conjugates (Scheme 18).

The authors used a classical Merrifield support, which represents a chloromethylated styrene–divinylbenzene (2%) copolymer. The synthesis of the peptide fragment was carried out using Boc-protected amino acids and *N*,*N*′-dicyclohexylcarbodiimide (DCC) as a condensation reagent. The choice of amino acids was determined so as to avoid complications resulting from the splitting of the target product off the polymer. The first nucleotide was attached to the peptidyl polymer by the so-called pyrophosphate method, which consists in the activation of the phosphate group of the nucleotide owing to the formation of mixed anhydride with diphenyl phosphate. This reaction was carried out in dry DMF in the presence of a tenfold excess of thymidine 5′-phosphate–diphenyl phosphate mixed anhydride (20 °C, 100 h). The elongation of the oligonucleotide chain was carried out by the phosphodiester method. The condensation was performed in dry pyridine in the presence of a five- or a tenfold excess of 3′-*O*-acetylthymidine 5′-phosphate and a fivefold excess of dicyclohexylcarbodiimide with respect to thymidylyl-(5′→N)-Phe-Gly-Phe. The deprotection of the 3′-hydroxy group of the nucleotide was carried out in alkaline aqueous dioxane (pH 9). Such a treatment ensured a practically complete removal of acetyl protection without splitting of the dinucleotidyl-(5′→N)-peptide off the polymer.

The final step included hydrolysis of the ester bond between the polymer and the trinucleotidyl-(5′→N)-peptide. The authors encountered serious difficulties because of both steric hindrances and the impossibility of using strong acids, which might result in cleavage of the phosphoramide bond. The maximum extent of cleavage of the ester bond (10%) is achieved by treating the oligonucleotidyl-(5′→N)-peptide with NaOH in aqueous dioxane.

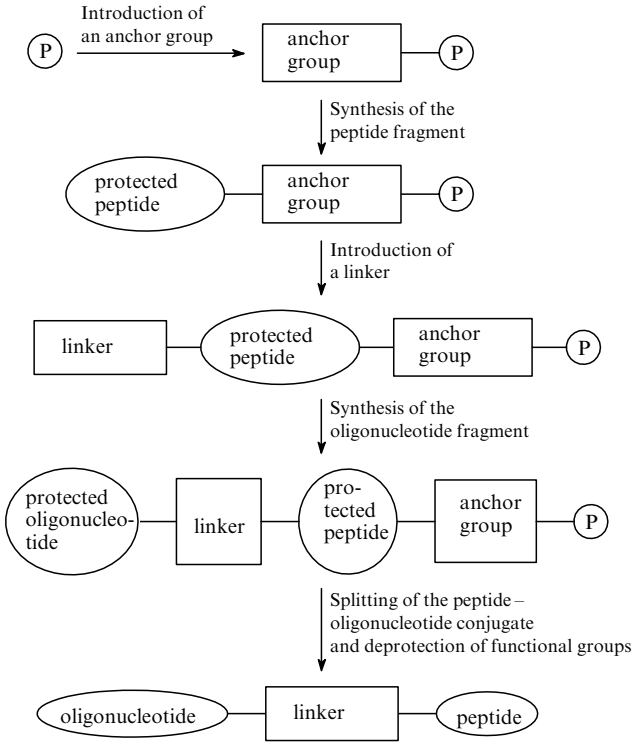
In its modern modification, this approach combines stepwise synthesis of the peptide from the C-end and subsequent elongation of the oligonucleotide chain on the peptidyl polymer by the phosphoroamidite method (Scheme 19). If the peptide is devoid of hydroxyamino acid residues, the synthesis of the DNA fragment should be preceded by incorporation of a hydroxy group into the peptidyl polymer. For this purpose, the α-amino group is acylated by a bifunctional reagent which contains an activated

Scheme 18



carboxy and a protected hydroxy group. This promotes the insertion of a linker between the N-end of the peptide and the oligonucleotide.

Scheme 19



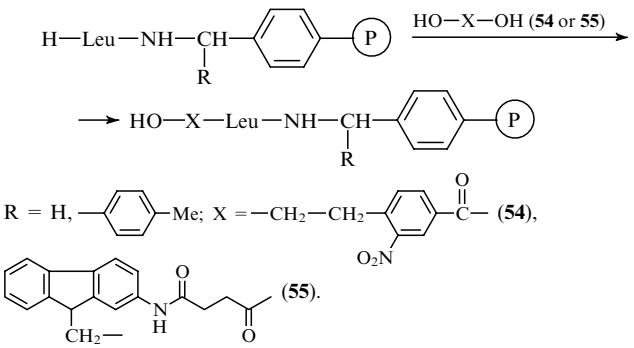
a. Solid support

The success of a solid-phase synthesis largely depends on the correct choice of solid support. The most popular support is controlled pore glass (CPG).[95–102] It is common practice to use CPG with a pore diameter of 500 Å. The synthesis of peptide – oligonucleotide conjugates on silica gel (Fractosil) has also been described.[100] However, in a more recent publication [102] the same authors had to admit that the use of CPG was preferable, since this solid support allows the synthesis of the target product in higher yields even at lower specific loading with the first monomeric unit. At the same time, some investigators (see, *e.g.*, Refs 103 – 105) call into question the expediency of CPG application, since the synthesis of the peptide fragment on this support affords products with shorter chains in addition to the target oligomer. In the authors' opinion,[53, 103] the formation of short peptide chains can be avoided through the use of a copolymer of polyethylene glycol with polystyrene (PEG-PS). In a series of publications by Spanish investigators,[104–113] the use of a copolymer of styrene with 1% divinylbenzene has proved to be especially efficient in peptide synthesis.

b. Introduction of an anchor group

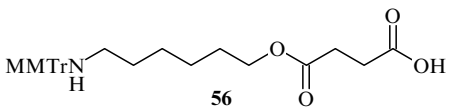
The first amino acid, which is to become a C-terminal residue in the peptide fragment to be synthesised, is attached to the support through the ester bond. In order to effect the esterification reaction between the amino acid and the solid support, the latter should contain reactive groups. Special methods for chemical modification of the support are being developed.

The use of polystyrene modified by aminomethyl [106–109] or *p*-methylbenzhydrylamine [109, 110] groups as a polymeric support is reported. In the first step, the NH_2 group of the support was acylated by *N*-protected leucine used as an internal standard. After removal of the amino protective group, the leucyl polymer was treated with a bifunctional reagent, *viz.*, 3-nitro-4-(2-hydroxyethyl)benzoic acid (**54**) [106–110, 113] or *N*-(9-hydroxymethylfluoren-2-yl) succinamide (**55**).[109]



The presence of a bridge between the support and the C-terminal amino acid residue allows splitting of the target product from the solid phase under very mild conditions under the action of base. The linkers can be attached directly to the support, *e.g.*, to PEG-PS containing an amino group.[53, 103]

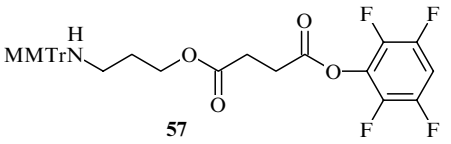
The use of *N*-protected 6-aminohexyl succinate **56** as a linker has been described.[53]



56

Commercially available CPG containing 3-aminopropyl groups was used [95–97, 100–102] for the synthesis of peptide – oligo-

nucleotide conjugates. In this case, chemical modification of the solid support was reduced to the acylation of 3-aminopropyl groups by activated esters of 4-hydroxybutyric [95–97] or 10-hydroxydecanoic acid [100–102] with the hydroxy groups protected as acid-labile dimethoxytrityl and pixyl (9-phenyl)xanthen-9-yl derivatives. Two additional ε-aminocaproic acid molecules were introduced between the 4-hydroxybutyric acid residue and the aminopropyl support.[97] Lukhtanov *et al.*[99] used porous glass modified by an extended linker containing a terminal NH_2 group. The latter was acylated with the activated ester **57**.



57

It is of note that the addition of the first amino acid to the support and the formation of the amide bond in the course of solid-phase synthesis of peptide fragments are usually carried out by the methods involving carbodiimide, activated esters or symmetrical anhydrides.

c. Protective groups

Temporary protection of α-amino groups of amino acids is mainly accomplished using Boc [53, 99, 101–103, 106-110] or Fmoc [95–98, 100] groups. Sequential solid-phase synthesis of peptide and oligonucleotide fragments demands protection of reactive side groups of certain amino acid residues. Protective groups are selected in order to ensure selective removal of other groups. The Fmoc [53, 101, 103, 104] or the Boc group [95, 96] are most commonly used for the protection of ε-amino groups of lysine; trifluoroacetyl (Tfa) [104] and 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde) [100] protective groups are used less frequently. The latter withstands splitting of the N^{α}-Fmoc group by treatment with 20% piperidine in DMF. The β-carboxy group of aspartic acid is protected by conversion into 9-fluorenylmethyl ester.[53, 103, 106, 108] The possibilities for the protection of the indole (in) ring of tryptophan are rather limited. N^{in}-Formyl protection is one of them. It is stressed [104] that the use of this protection in the synthesis of peptide–oligonucleotide conjugates prevents the formation of side product. Marchan *et al.*[104] have succeeded in obtaining Boc-Arg(Fmoc)$_2$-OH which can be used in the solid-phase synthesis of the peptide fragment of the conjugate.

It is known[111] that unprotected amide groups of asparagine or glutamine residues in the peptide fragments of conjugates can undergo phosphitylation during subsequent elongation of the oligonucleotide chain. However, this side reaction proceeds at a very low rate which excludes the necessity of blocking side groups of asparagine and glutamine.[53, 104]

Grandas *et al.*[109, 110, 112] protected the hydroxy group of homoserine by the dimethoxytrityl group leaving the HO groups of serine, threonine and tyrosine unprotected.[106–110] Other authors [101] recommend protecting the hydroxy groups of serine as allyl carbonates. Assuming that the use of the Fmoc-strategy of solid-phase peptide synthesis may result in partial cleavage of this protective group, the authors gave preference to the Boc-strategy.

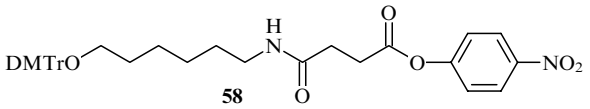
Despite the vast variety of techniques for the protection of the imidazole (im) ring of histidine, no ideal protective group has been found for this compound. In the synthesis of peptides containing this amino acid, N^{im}-benzyloxymethyl protection was used and deprotection was effected by catalytic hydrogenolysis over palladium black in the presence of cyclohexa-1,4-diene. According to Truffert *et al.*,[102] such a treatment should not have any negative effect on the stability of the oligonucleotide fragment of the conjugate. However, this method can hardly be considered satisfactory, since the authors failed to completely remove the peptide–oligonucleotide conjugate formed from the catalyst's surface.

The use of the Boc or the Fmoc groups for the protection of the imidazole ring of histidine cannot be recognised as very convenient either. The reason is that treatment of the peptide–oligonucleotide conjugate with trifluoroacetic acid to remove the N^{im}-Boc group can result in hydrolysis of the N-glycosidic bonds in purine nucleotides, whereas the N^{im}-Fmoc group is rather labile under conditions of the solid-phase synthesis. In the authors' opinion,[102] the tosyl (Tos) group is especially convenient for the protection of the imidazole ring of histidine. This group is removed under the action of 1-hydroxybenzotriazole commonly added to the reaction mixture in order to inhibit racemisation in the formation of the peptide bond promoted by DCC. Two procedures for the protection of the imidazole ring of histidine by the tosyl and 2,4-dinitrophenyl groups have been developed by Beltrán *et al.*[110] These authors share the opinion that 1-hydroxybenzotriazole can be replaced by tetrazole which has proved to be an efficient reagent when combined with DCC. The analysis of reversed-phase HPLC data led the authors to conclude that the use of Boc-His(Tos)-OH and deprotection of the imidazole ring immediately prior to the elongation of the oligonucleotide chain is the most efficient approach to the solid-phase synthesis of the peptide fragment.

d. Introduction of the linker

After termination of solid-phase synthesis of the peptide component of the conjugate containing a hydroxy amino acid residue,‡ deprotection of the α-amino group is followed by its repeated selective protection by introducing the phenylacetyl (Pac) [106–108, 113] or the acetyl (Ac) group [109, 110] after which the oligonucleotide synthesis is performed (Scheme 20).

If the peptide fragment of the conjugate is devoid of hydroxy amino acids, deprotection is followed by acylation of the free α-amino group by a compound containing an activated carboxy group and a protected hydroxy group.[53, 95–103] *p*-Nitrophenyl *O*-pixyl-4-hydroxybutyrate,[97, 99] *p*-nitrophenyl [100, 101] and pentafluorophenyl [102] *O*-dimethoxytrityl-10-hydroxydecanoates and compound **58** [53, 95, 96, 103] are used as acylating reagents.

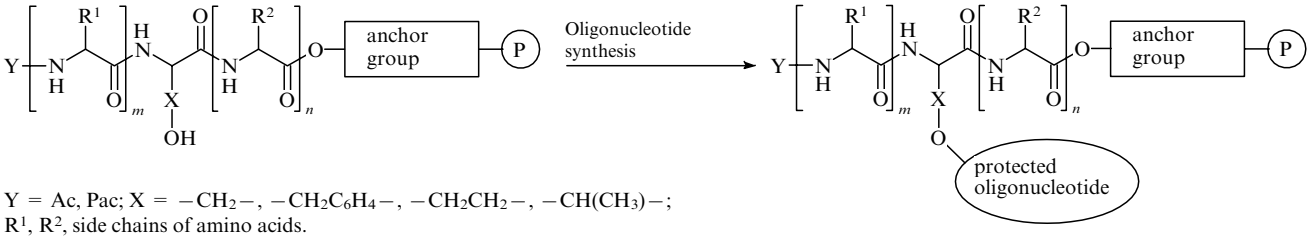


58

The incorporation of the aliphatic OH group into the peptide and its further selective deprotection make the synthesis of the DNA fragment possible.

‡ For incorporation of homoserine into the peptide chain, N^{α}-Pac-*O*-DMTr-Hse is introduced into the condensation reaction in the form of the triethylammonium salt.[109, 110]

Scheme 20



Y = Ac, Pac; X = $-CH_2-$, $-CH_2C_6H_4-$, $-CH_2CH_2-$, $-CH(CH_3)-$;
R^1, R^2, side chains of amino acids.

e. Synthesis of the oligonucleotide fragment

In the studies [95–102] where CPG was used as a solid support, automated solid-phase phosphoramidite synthesis of an oligonucleotide component of a conjugate was carried out according to a standard protocol. If a copolymer of styrene with ethylene glycol [53, 103] or a copolymer of styrene with 1% divinylbenzene [106–113] are used as polymeric supports, the standard procedures had to be modified in order to increase the yields in each step of the oligonucleotide synthesis. These modifications include a severalfold increase in the time required for implementation of all the main steps of internucleotide bond formation, the use of 5-(2-nitrophenyl)tetrazole instead of tetrazole and a significant increase in the concentration of the nucleotide solution in dichloromethane.

In the overwhelming majority of studies, the elongation of the oligomeric chain was carried out in the direction 3′→5′ except for the cases where 5′-phosphoramidites of protected deoxynucleosides were used as monomeric components.[106–108] Labile protective groups [106, 108, 109, 113] were used together with acyl ones for the protection of exocyclic amino groups of heterocyclic bases. For instance, the *N*,*N*-dimethylformamidine and *tert*-butylphenoxyacetyl groups were used for the protection of the NH_2 groups of adenine and guanine, while the isobutyryl and the *tert*-butylphenoxyacetyl groups were used for cytosine.

f. Cleavage of conjugates from the solid support and deprotection of functional groups

The problem which has to be solved for the successful solid-phase synthesis of peptide – oligonucleotide conjugates by stepwise elongation of peptide and oligonucleotide chains is to find optimal conditions for the deprotection of functional groups and cleavage of the bonds between the conjugate and the support. Thus it is not recommended to liberate the conjugate from the support by treatment with strong acids or by using acid-labile protective groups because of the risk of partial apurinisation of DNA. For this reason, in the majority of studies post-synthetic treatments were carried out under mild basic conditions. The apprehensions that alkaline treatment might lead to racemisation of the amino acid residues at the α-carbon atom were not confirmed.[53] It was noted also that alkaline treatment of conjugates in which the peptide is linked to the oligonucleotide through a serine residue favours the cleavage of the phosphodiester bond between the two fragments.[3,4] This reaction includes cleavage of the C–O bond and occurs as base-catalysed β-elimination (see Scheme 2). However, a detailed analysis of this problem showed [108] that treatment of conjugates with a concentrated aqueous ammonia – dioxane mixture (1 : 1) at room temperature for 15–24 h did not cause the cleavage of the bond between the oligonucleotide fragment and the serine residue.

A convenient procedure was developed [106–109] for the liberation of peptide – oligonucleotide conjugates from the solid phase by treatment with 0.05 M tetrabutylammonium fluoride in tetrahydrofuran at room temperature for 30–60 min. Such treatment ensures simultaneous removal of β-cyanoethyl protective groups from internucleotide phosphates and splitting of the 9-fluorenylmethyl ester of aspartic acid. This is followed by deprotection of exocyclic amino groups of heterocyclic bases under the action of a mixture of concentrated aqueous ammonia and dioxane (1 : 1) at room temperature for 15–18 h. The latter procedure was also carried out at 55 °C (see Ref. 110). Apparently, more drastic conditions were required for the removal of the isobutyryl protective group from the exocyclic amino group of the guanine base in the DNA fragment. The same procedure was used for the removal of some other protective groups, *e.g.*, the tosyl and the 2,4-dinitrophenyl groups (from histidine).

However, an attempt of de la Torre *et al.*[53] to liberate a peptide – oligonucleotide conjugate from the polymeric support (PEG-PS) using tetrabutylammonium fluoride failed. It was assumed that polyethylene glycol, which is a constituitive element of the support, prevents β-elimination catalysed by the fluoride ion. The use of tetrabutylammonium fluoride is also unacceptable for the liberation of those peptide – oligonucleotide conjugates in which the components of the conjugate are linked through the hydroxy group of tyrosine. In this case, the two parts of the hybrid molecule are linked through a phosphotriester bond. It is therefore quite probable that the nucleophilic attack of the fluoride ion at the phosphorus atom will be accompanied by the cleavage of the P–O bond with the formation of a stable phenoxide ion. Therefore, Robles *et al.*[109] proposed to use a mixture of concentrated aqueous ammonia and dioxane (1 : 1) at 55 °C for 18 h for the liberation of the peptide – oligonucleotide conjugate from the solid support. It should be noted that the carboxy group of the C-terminal amino acid is converted into the amide group under these conditions.

The use of concentrated aqueous ammonia for the cleavage of peptide – oligonucleotide conjugates from the solid support and concomitant removal of protective groups yielded a mixture of products.[53, 96] This can be explained by the fact that the cleavage of the ester bond under the action of concentrated ammonia is accompanied by the formation of both an amide and an ammonium salt. Prior to ammonolysis, the oligonucleotide – peptidyl polymer was treated with a solution of 0.5 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in acetonitrile.[53] This made it possible to remove the N^{ε}-Fmoc and β-cyanoethyl groups and to cleave the 9-fluorenylmethyl ester of aspartic acid. In the authors' opinion, this reaction order should exclude the conversion of the ester into the amide.

In order to liberate the protected peptide – oligonucleotide conjugate from the support, Truffert *et al.*[100] used a 50% solution of ethanolamine in absolute ethanol (60 °C, 30 h). The final product was obtained in the form of monoethanolamide in a very low yield. To increase the yield, Truffert *et al.*[101, 102] used 0.1 M NaOH instead of ethanolamine. Under these conditions, hydrolysis of the ester bond between the peptide – oligonucleotide conjugate and the support is completed in 2 h at room temperature and is accompanied by a simultaneous removal of other protective groups, *e.g.*, the β-cyanoethyl groups from internucleotide phosphates, the tosyl group from histidine, the allyloxycarbonyl group from serine and the Fmoc or Dde groups from lysine. The authors note that deprotection of exocyclic amino groups of adenine, cytosine and guanine requires no less than 24 h.

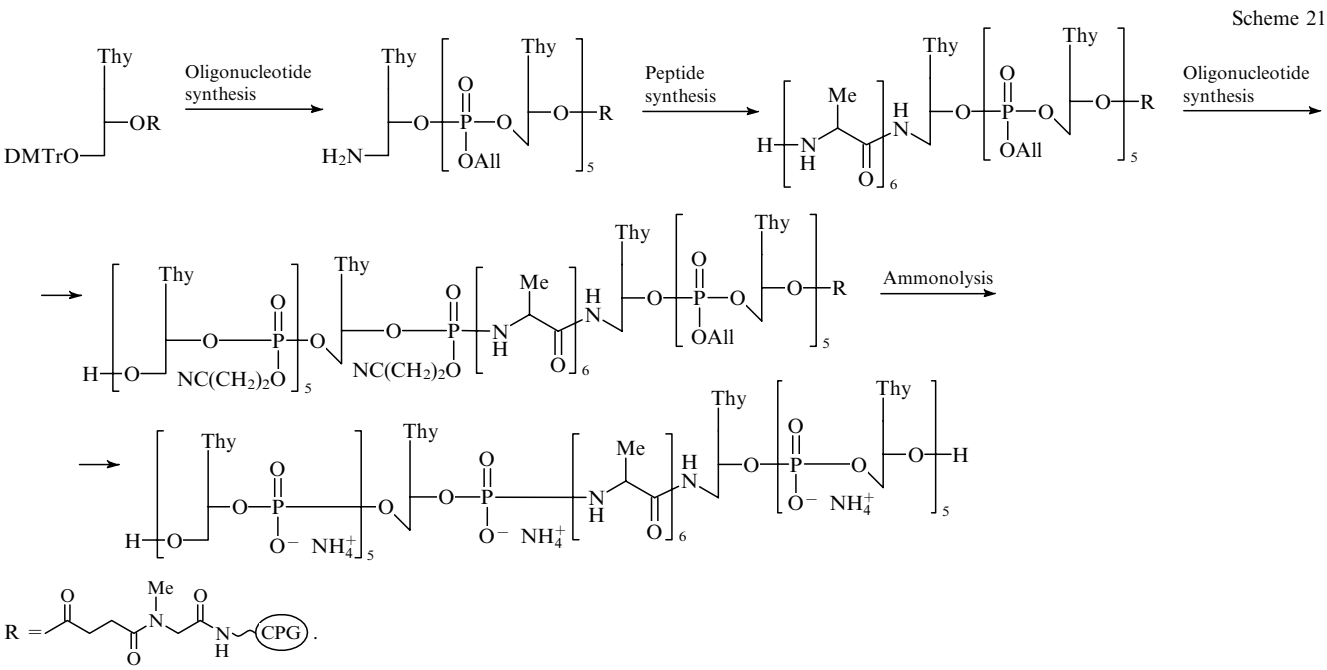
The use of the Boc-protection for the side amino group of lysine is undesirable, since its removal proceeds under rather drastic conditions [treatment with trifluoroacetic acid and 1,2-ethanedithiol (9 : 1) for 5 min [95, 96]] which may lead to hydrolysis of *N*-glycosidic bonds in the oligonucleotide fragment and subsequent destruction of the target product.

2. Sequential synthesis of oligonucleotide and peptide fragments on the same solid support

In the studies considered above, peptide – oligonucleotide conjugates were prepared by stepwise synthesis, *viz.*, the peptide fragment of the hybrid molecule was first synthesised on a solid phase after which it was possible to proceed to the synthesis of the oligonucleotide chain. In an alternative approach, the DNA fragment is synthesised first. The main limitation of this method is the impossibility of using the Boc-strategy for the peptide synthesis.

The solid-phase synthesis of oligonucleotides with a 5′-terminal reactive NH_2 group has been described.[114–117] To this end, the 5′-amino-5′-deoxythymidine residue was incorporated into the oligomeric chain in the final step of automated phosphoramidite synthesis. The nucleoside thus modified plays the role of a linker between the oligonucleotide and the peptide fragments of the conjugate. The Fmoc-strategy was used for the elongation of the peptide chain by Bergmann and Bannwarth [114] (Scheme 21); the activation of the carboxy group was carried out *in situ* using *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′*N*′-tetramethyluronium hexafluorophosphate (HBTU). The allylic group was selected as the phosphate-protective group instead of the β-cyanoethyl group

Scheme 21



commonly used for this purpose, because in this study the Fmoc-group was removed by a strong base (DBU) instead of piperidine. For the same reason, the solid-phase synthesis was carried out on modified CPG. To ensure the stability of the bond between the oligonucleotide and the support during DBU treatment, a sarcosine residue was incorporated into the anchor group.

After termination of the synthesis of the peptide fragment, synthesis of another oligonucleotide chain was begun with the use of 2-cyanoethyl *N*,*N*-diisopropylphosphoramidites of 2′-deoxynucleosides. This procedure afforded conjugates in which two DNA fragments were linked through a peptide fragment. These were liberated from the solid support by consecutive treatment with $Pd[PPh_3]_4$ and morpholine in a DMSO–THF–dioxane–0.5 M HCl mixture (2:2:2:1) at 60 °C for 3 h and then with 30% aqueous ammonia at room temperature for 2 h. It should be noted that the bond between the peptide and the second oligonucleotide is of a phosphoramide type, which is not cleaved in the post-synthetic deprotection of the peptide–oligonucleotide conjugate and its liberation from the solid support.

An analogous synthetic scheme was developed for the preparation of conjugates in which the oligonucleotide fragment is a heteropolymer.[115,116]

Special mention should be made of one of the most recent studies carried out by Bleczinski and Richter [117] which describe a procedure for the preparation of cyclic peptide–oligonucleotide conjugates. Fmoc-protected amino acids and All-protected nucleoside phosphoramidites were used as the starting compounds. This approach was used to obtain the cyclic hybrid Glu-Leu-T*T-DP-Lys (**59**) (T* is 5′-amino-5′-deoxythymidine, DP is 3-hydroxy-2,2-dimethylpropionic acid). The amide bond of the 'head-to-tail' type was formed by the ε-amino group of lysine and the γ-carboxy group of glutamic acid. The structure of compound **59** was confirmed by ^{1}H NMR spectroscopy and MALDI-TOF mass spectrometry. The cyclic conjugate appeared to be resistant to exo- and endonucleases. By varying the peptide fragment, it was possible to obtain an analogous 39-membered ring.

3. Synthesis of peptide–oligonucleotide conjugates on solid supports with a bifunctional branched anchor group

An approach to the synthesis of peptide–oligonucleotide conjugates proposed in the early 1990's [118–121] and further successfully developed in the studies carried out in the past three years [122–125] is of considerable interest. The oligonucleotide syntheses were performed on solid supports with branched anchor groups containing HO and NH_2 groups. The presence of this group allows sequential attachment of monomeric units, first, of one type and then, of another type, on the same support. Post-synthetic treatments result in a hybrid molecule in which the oligonucleotide and the peptide fragments are connected through a linker. The distinctive feature of this method is that the bifunctional § linker is attached directly to the solid phase rather than to one of the fragments of the synthetic conjugate. 3-Aminopropane-1,2-diol [118] and 6-amino-2-hydroxymethylhexan-1-ol [119] can be used as linkers. The hydroxy and amino functions are protected by the DMTr and Fmoc groups commonly used for this purpose.

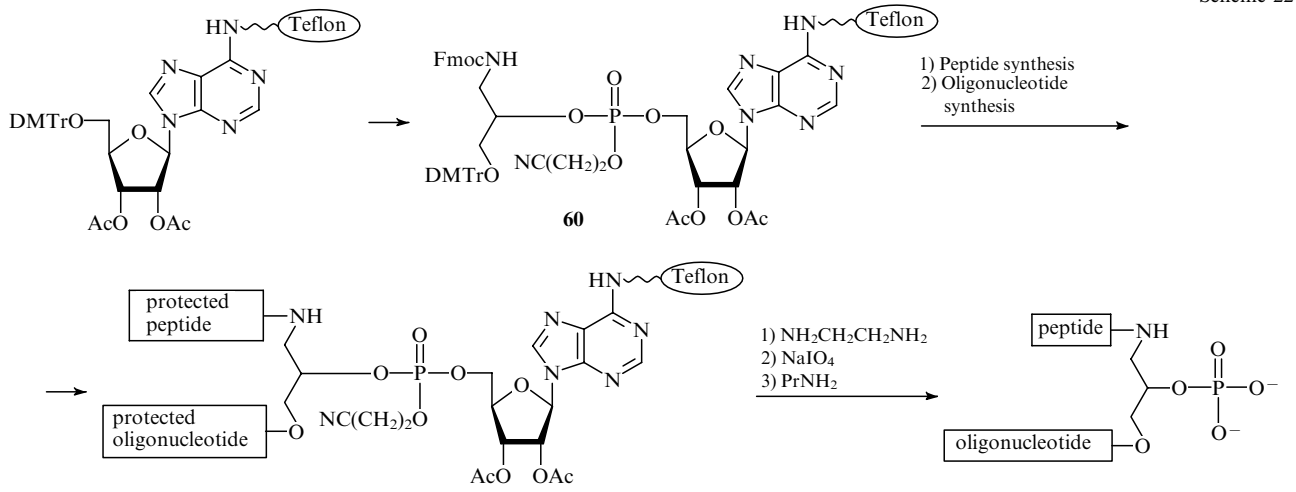
In these studies,[118,119] the peptide fragment was synthesised first. In the peptide synthesis, the ε-amino group of lysine was protected by the Boc group; the hydroxy group of serine and the thiol group of cysteine were protected by the *tert*-butyldimethylsilyl (TBDMS) and trityl (Tr) groups, respectively, whereas the amino groups of adenine and guanine were protected by the 2-(acetyloxymethyl)benzoyl group, while those of cytosine were protected by the acetyl group.

The main difference in the approaches proposed by Canadian [118] and American [119] investigators consists in the choice of an insoluble support and the method for its chemical modification.

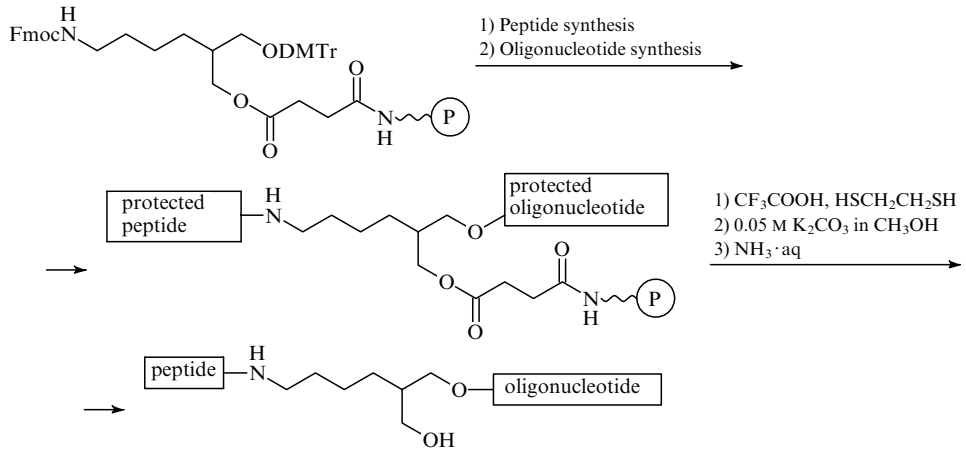
The polymer employed by the Canadian investigators Juby *et al.*[118] was commercially available Teflon to which 5′-*O*-dimethoxytrityl-2′,3′-di-*O*-acetyladenosine was attached through an extended linker. The first step was the selective removal of the DMTr protective group from the 5′-hydroxy group followed by the introduction of a protected 3-aminopropane-1,2-diol residue using the amidite strategy to yield derivative **60** (Scheme 22). This was employed for the peptide and then for the oligonucleotide synthesis. After completion of the peptide and oligonucleotide syntheses, the functional groups were deprotected under mild conditions by treatment with ethylenediamine in absolute ethanol (1:1) at 55 °C for 1 h. (This procedure for the removal of protective groups was specially developed in the synthesis of oligonucleotide methylphosphonate derivatives.) The cleavage of the target product from the solid phase was carried out in two steps. At first, the *cis*-glycol group of the 2′,3′-di-*O*-acetyl

§ It would be more correct to speak about a tri- rather than about a bifunctional linker, since it contains one more reactive group involved in the formation of a covalent bond with a solid support.

Scheme 22



Scheme 23

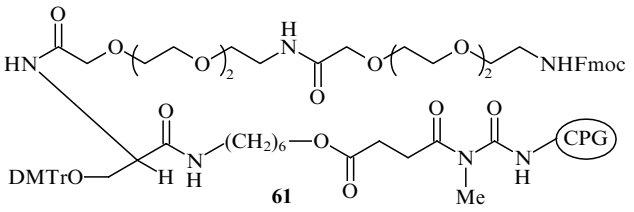


adenosine linker was oxidised by sodium periodate after which β-elimination of the peptide – oligonucleotide conjugate [cleavage of the C(5′)–O bond] under the action of $PrNH_2$ was performed.

The American investigators Basu and Wickstrom [119] used a linker-modified copolymer of styrene with ethylene glycol as a support (Scheme 23).

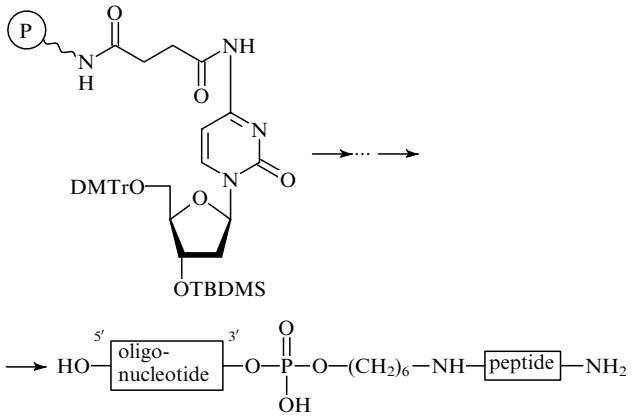
The post-synthetic deprotection of functional groups in the target product was performed under mild basic conditions (0.05 M K_2CO_3 in methanol). It was noted that complete cleavage of the bond between the peptide – oligonucleotide conjugate and the polymer required additional 30-min treatment with ammonia at room temperature.

Successful application of the solid support **61** with a branched linker for the synthesis of peptide – oligonucleotide conjugates has been reported by Nielsen *et al.*[121]

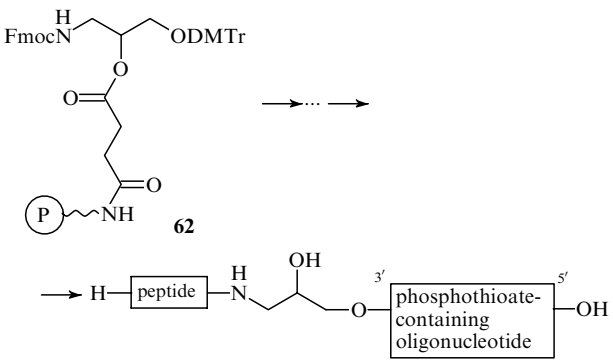


De Napoli *et al.*[122] used a nucleoside residue containing cytosine as a heterocyclic base for the modification of the polymeric support. This residue was attached to the spacer group of the polymeric support through the exocyclic amino group of cytosine (Scheme 24). The differently protected 3′- and 5′-hydroxy groups of the deoxyribonucleoside residue on the polymeric support were subject to subsequent modifications by phosphoramidite derivatives. The 5′-hydroxy group was used for conventional construction of the oligonucleotide chain in the direction 3′→5′, whereas the 3′-hydroxy group was phosphitylated by a commercially available standard amino linker and subsequently used for peptide synthesis.

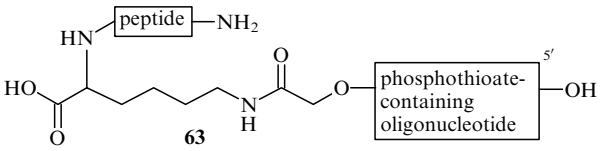
Scheme 24



The design, synthesis and testing of a novel polymeric support **62** for the synthesis of peptide – oligonucleotide conjugates and their phosphothioate analogues have been described.[123, 124] The deblocked hydroxy group of this support was involved in standard oligonucleotide syntheses, while the deblocked amino group is involved in peptide synthesis. As a result, the 3′-end of the oligonucleotide becomes bound to the C-end of the peptide through a phosphodiester (or a thiophosphodiester) bond and a short linker.



Later, the same authors [125] performed the synthesis of the peptide – oligonucleotide conjugate **63** using a polymeric support with a branched linker.



4. Synthesis of oligonucleotide peptides by fragment condensation

The essence of this method is in the formation of a covalent bond between the presynthesised peptide and the oligonucleotide after completion of the automated oligonucleotide synthesis but prior to the cleavage of the nucleic acid fragment from the solid support. The main advantage of this approach is that one component of the conjugate is not exposed to the effect of chemical reagents used for the synthesis of the other component as is the case with sequential solid-phase synthesis of peptide – oligonucleotide conjugates.

Very often, peptide – oligonucleotide conjugates are prepared by the reaction of an oligonucleotide containing free 5′-hydroxy groups and immobilised on an insoluble support with a peptide phosphoramidite derivative in the presence of tetrazole.[108, 111, 126–133] The peptide phosphoramidite derivatives can be prepared in good yields by the reaction of the hydroxy group of serine or tyrosine with a standard phosphitylating reagent.[108, 126–132] It was also proposed to phosphitylate the C-terminal amide group of the peptide.[111, 133]

As in the case of other methods for the synthesis of peptide – oligonucleotide conjugates, the main efforts of investigators were directed at a search for the mildest conditions for post-synthetic treatment. This is especially important in those cases where the formation of the phosphodiester bond between the oligonucleotide and the peptide fragments of the conjugate involves the serine residue. Such compounds destroy under alkaline conditions. In contrast, those molecules, which contain phosphoramide bonds, are unstable in strongly acidic media.

van Boom *et al.*[128] protected the α-amino group of the peptide by the *p*-nitrobenzyloxycarbonyl group and the ω-carboxy groups of aspartic and glutamic acids were converted into *p*-nitrobenzyl esters. These protective groups are removed simultaneously with a sodium dithionite – sodium hydrogencarbonate mixture. The exocyclic amino groups of the heterocycles were protected by the 2-(*tert*-butyldiphenylsilyloxymethyl)benzoyl group. Oxalic acid served as a linker between the first nucleoside and the polymer.[128] Liberation of the target product from the solid phase with simultaneous deprotection of heterocyclic bases and internucleotide phosphates was carried out using tetrabutylammonium fluoride in aqueous pyridine.

It is known that allyloxycarbonyl and allyl protective groups are split off under mild conditions. These groups were used [129–131] for the protection of the functional groups of the oligonucleotide (exocyclic amino groups of heterocyclic bases, internucleotide phosphates) and the peptide (terminal NH_2 and COOH groups) fragments. These protective groups are easily removed by a palladium complex in the presence of diethylammonium formate.

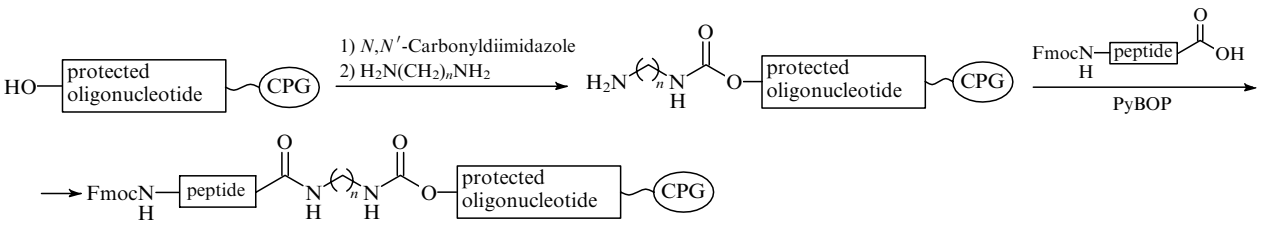
The synthesis of peptide – oligonucleotide conjugates containing nucleotidyl-(5′→O)-serine fragments was performed using protection of the lateral carboxy group of aspartic acid by any of the three esters, *viz.*, 9-fluorenylmethyl, benzyl or 2-(2-nitro-4-acetylphenyl)ethyl esters.[108] The exocyclic amino groups of adenine residues were protected by benzoyl or *N*,*N*-dimethylformamidino group. The cleavage of the peptide – oligonucleotide conjugate from the solid support and removal of protective groups were carried out using three different methods, *viz.*, by treatment with concentrated aqueous ammonia, potassium carbonate or lithium hydroxide in a methanol – dioxane mixture. It is of note that the authors [108] failed to correctly characterise the target product and to confirm its structure by mass spectrometry and amino acid analysis data.

The methods for the synthesis of conjugates containing an amide bond between the oligonucleotide and the peptide fragments were considered in the section devoted to the solution synthesis of peptide – oligonucleotide conjugates (see Section II.3). The difficulties reported in the publications [38, 39] could be largely overcome by performing the solid-phase condensation of the oligonucleotide and the peptide components, which significantly increased the yield of the target product. The decomposition of diazoketone **37** (see Scheme 10) was carried out in the presence of silver benzoate rather than under UV irradiation in the apprehension that in the latter case the reaction rate would decrease due to partial absorption of energy by the solid support (polystyrene or CPG).[39]

The condensation of the pentapeptide Ac-Ala-Tyr-Gln-Val-Phe with tri- and tetranucleotides containing 5′-terminal 5′-amino-5′-deoxythymidine residues and immobilised on a solid phase was reported by Tetzlaff *et al.*[115] However, these authors drew attention to the low efficiency of this reaction, which they attributed to the low solubility of the peptide in dimethylformamide.

The recently published data deserve special mentioning. Thus, Gait *et al.*[134, 135] used the following approach for the incorporation of an aliphatic amino group into the DNA fragment: the 5′-hydroxy group of the CPG-linked oligonucleotide following removal of the terminal DMTr protective group was activated by *N*,*N*-carbonyldiimidazole and introduced into the reaction with a diamine (Scheme 25). When PyBop was used as a condensation reagent in the presence of 1-hydroxybenzotriazole, the degree of racemisation at the α-carbon atom of the C-terminal amino acid was very small. Nevertheless, peptides containing C-terminal

Scheme 25



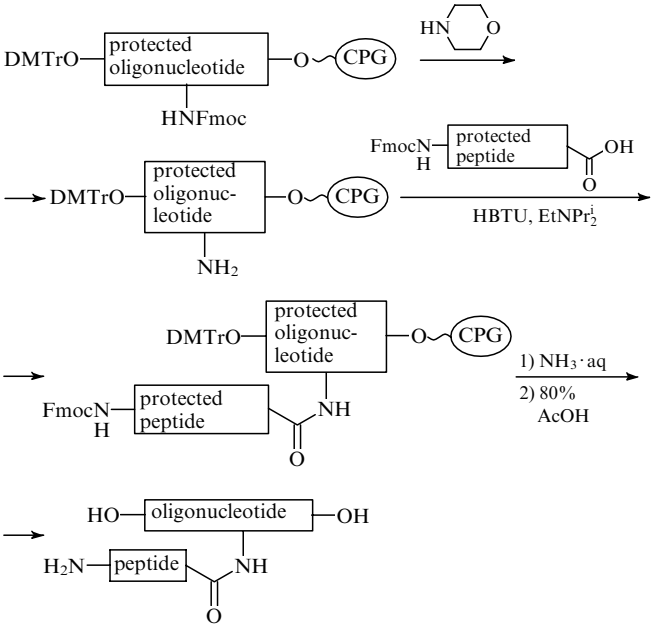
n = 3, 6, 12.

glycine were selected for conjugation to oligonucleotides, since in this case the α-carboxy group of the peptides could be activated without any risk of racemisation. A tetrapeptide containing nonpolar amino acid residues and an octapeptide built up of polar amino acids were used as peptide fragments, while various homo- (*e.g.*, T_{12}) and heterooligonucleotides were used as oligonucleotides. The conjugation of both peptides to T_{12} proceeded with high efficiency.[134, 135] However, the yields of peptide – oligonucleotide conjugates prepared by condensation with heterooligonucleotides were very low. The authors failed to explain the reason for this phenomenon. Neither did the changes in experimental conditions, *i.e.*, the use of PEG-PS instead of CPG, a larger peptide excess (10 instead of 5 equiv.) and higher temperature (40 °C), give any positive result.

It was suggested that the main problem is related to poor solubility of protected heterooligonucleotides in dimethylformamide due to their high hydrophobicity. This problem was overcome by selective removal of β-cyanoethyl protective groups by triethylamine in acetonitrile which caused some increase in the yields of the target products. In the majority of cases, the elongation of the linker containing the amino group also had a beneficial effect on the conjugate yields.

A modification of this reaction was employed[136] in the condensation of a presynthesised peptide with a blocked oligonucleotide immobilised on a solid phase and containing a linker attached to the C(2′)-atom of the carbohydrate fragment (Scheme 26). Oligodeoxyribonucleotides employed contained either three modified nucleosides, *viz.*, 2′-(3-aminopropionyl)-amino-2′-deoxy-*arabino*-adenosine, 2′-(3-aminopropionyl)amino-2′-deoxyuridine or 2′-amino-2′-deoxy-*arabino*-adenosine.

Scheme 26



First, the Fmoc-protected amino group attached to the C(2′)-atom of the carbohydrate fragment of one of nucleotide residues either directly or through a linker was selectively deprotected with 50% morpholine in dimethylformamide for 1 h. Then the oligonucleotide with the free NH_2 group reacted with a protected peptide fragment.

Two short peptides, *viz.*, N^{α}-Fmoc-Leu-Gly and N^{α}-Fmoc-Tyr-D-Ala-Phe-Gly, were selected as peptide fragments. In both cases, the C-terminal amino acid residue was represented by a glycine residue. The latter circumstance is very important, since in this case the activation of the carboxy group of the peptides was not accompanied by racemisation due to the absence of a chiral carbon atom in the glycine molecule.

The reaction of the N^{α}-blocked peptide with the oligodeoxyribonucleotide immobilised on CPG-500 was carried out in anhydrous dimethylformamide in the presence of HBTU and ethyldiisopropylamine. The reaction was complete within 1 – 2 h and gave high yields (80% – 90%). In the final step, the conjugate was cleaved from the solid support and the functional groups of the oligonucleotide and the peptide components were deprotected.

The use of the solid-phase method requires large excesses of the peptides and the condensation reagent. This increases the efficiency of formation of the amide bond; the excesses of dimethylformamide-soluble reagents are easily removed by thorough washing of the support.

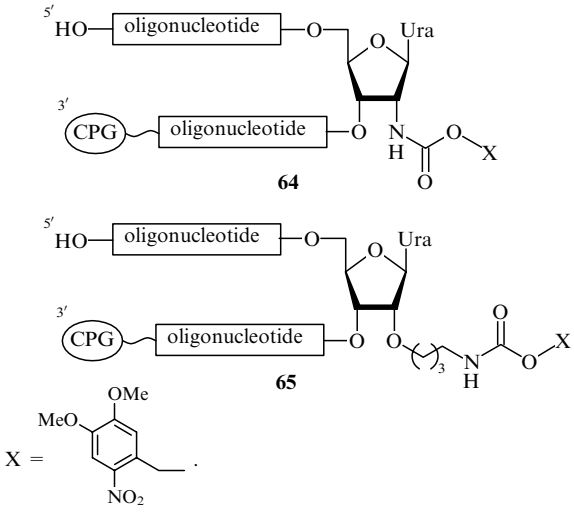
The main advantage of Fmoc protection of amino groups is that this can be removed in two ways. The first approach entails post-synthetic treatment of the oligonucleotide with saturated aqueous ammonia. The second approach consists of the removal of the Fmoc group with morpholine in an anhydrous medium, which allows selective deprotection of the aliphatic amino group of the oligonucleotide. Under these conditions, the oligonucleotide is not split from the support, and its other functional groups remain protected.

An approach proposed by Zubin *et al.*[136] allows simultaneous attachment of several peptide fragments to the oligonucleotide, since the modified nucleoside fragment containing an aliphatic amino group can be inserted into any predetermined position of the oligomeric chain and the number of inserts is not limited in principle. Another salient advantage of this approach is that the 5′- and 3′-ends of the oligonucleotide fragment of the conjugate remain free, which allows the attachment of various reporter groups, *e.g.*, radioactive or fluorescent labels.

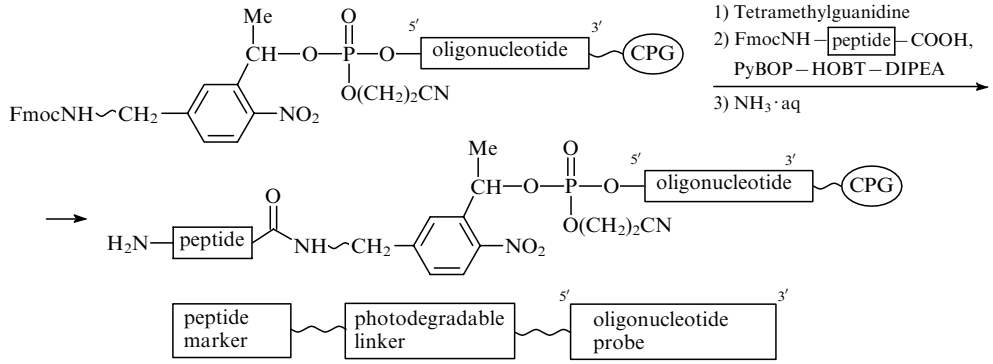
Certain difficulties arise when this method is used for the synthesis of oligonucleotide conjugates with larger peptides. The problem is associated with the activation of carboxy groups. Moreover, this method demands the use of large excesses of peptides, which is hardly justified from the economical point of view.

The approach based on successive attachment of small peptide fragments to oligonucleotides is an alternative. After attachment of the first fragment, the N^{α}-Fmoc group has to be removed, after which the next fragment is introduced into the reaction. N^{α}-Fmoc-Leu-Gly and N^{α}-Fmoc-Tyr-D-Ala-Phe-Gly were used for the synthesis of oligodeoxyribonucleotide conjugates with peptides containing four and eight amino acid residues.

A similar ideology is shared by Hwang and Greenberg[137, 138] who have succeeded in incorporating 2′-modified phosphoramidite synthons into different positions of the oligonucleotide chain in the course of automated synthesis. The amino group at the C(2′) atom of the synthon was protected by the photolabile protective group (X).

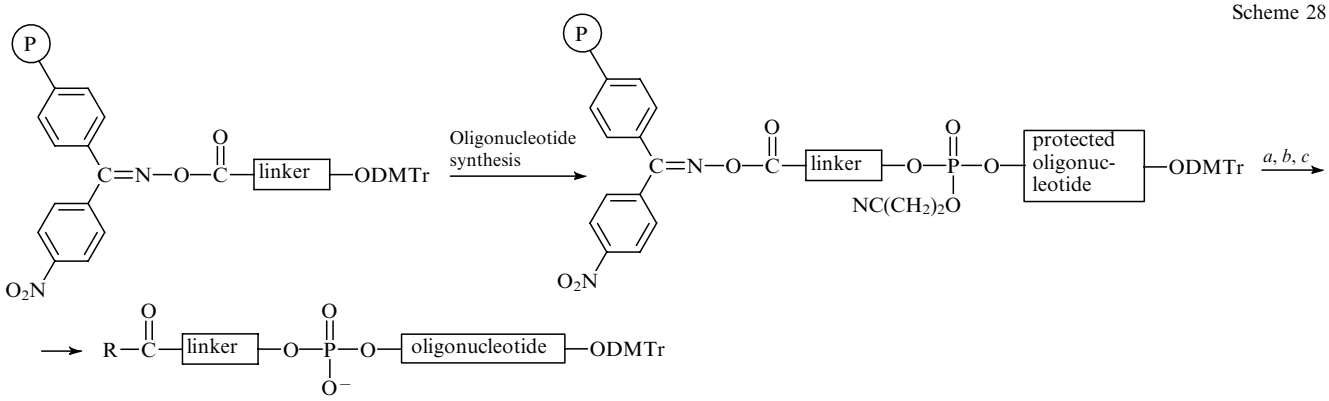


Scheme 27



DIPEA is diisopropylethylamine.

Scheme 28



Linker is $-(CH_2)_6-$, $-Gly-NHCO(CH_2)_6-$, $-Gly\text{-}Gly\text{-}Gly-NHCO(CH_2)_6-$;
(*a*) $EtNPr^i_2$; (*b*) RH (RH is H-Gly-Gly-Gly-NEt_2 or H-Leu-Ala-Lys(Tfa)-Leu-NEt_2); (*c*) $NH_3 \cdot aq$.

The attachment of tripeptides to the modified oligonucleotides **64** and **65** afforded the corresponding conjugates with satisfactory (70% – 80%) yields.

The synthesis of photodegradable peptide – oligonucleotide conjugates designed for detection of target NA sequences by the MALDI mass spectrometry methods has been described (Scheme 27).[139]

The photodegradable conjugate contains an oligonucleotide probe for target DNA or RNA sequences and a peptide mass marker easily detectable by mass spectrometry after hybridisation and subsequent photodegradation of the conjugate.

A special polymer with ketoxime functional groups was proposed for oligonucleotide synthesis (Scheme 28).[140, 141] The anchor group (linker) was attached to the support through an ester bond. The use of such supports facilitates the cleavage of the target product under the action of nucleophiles, the role of a nucleophile can be played by the peptide fragment.

IV. Conclusion

The analysis of published data suggests that no versatile method for the synthesis of peptide – oligonucleotide conjugates of any structure exists. This is due to the fact that several factors play crucial roles in the synthesis of peptide – oligonucleotide conjugates, *viz*., the type of modified nucleic acid fragment, the amino acid sequence, the size of the peptide, the conjugation procedure and the type of bond connecting the two parts of the hybrid molecule. The possibility of varying these parameters within a sufficiently broad range and of using their different combinations have given a strong impetus to the appearance of numerous publications devoted to novel methods for the synthesis of peptide – oligonucleotide conjugates. However, because of serious limitations of each of these methods, very few of them have found wide acceptance in the preparative synthesis of peptide – oligonucleotide conjugates. In this connection, a search for optimal approaches to the synthesis of peptide – oligonucleotide conjugates still remains a very urgent task.

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