Oligonucleotide-peptide conjugates as potential antisense agents

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Received 21 May 1999; received in revised form 11 June 1999

Abstract Oligonucleotide-peptide conjugates have several applications, including their potential use as improved antisense agents for interfering with the RNA function within cells. In order to provide robust and generally applicable conjugation chemistry, we developed a novel approach of fragment coupling of pre-synthesized peptides to the 2′-position of a selected nucleotide within an otherwise protected oligonucleotide chain attached to a solid support.

Key words: Antisense; Nucleic acid analogue; Oligonucleotide-peptide conjugate; 2′-Amino-2′-deoxynucleoside

1. Introduction

Considerable attention has been devoted recently to the design of oligonucleotide analogues to improve their antisense characteristics and eventually create therapeutic remedies of greatly increased efficiency. Examples include various modifications introduced at the base, sugar and phosphate moieties to attain nuclease resistance, enhanced duplex stability and cellular uptake [1]. Completely satisfactory solutions to all these problems are not yet available. Nevertheless, one can be optimistic that this research effort will bear fruit.

One general form of modification is to covalently attach a large variety of specific ligands to the oligonucleotides. Among the possible appendages are peptides, whose conjugation to DNA fragments produces oligonucleotide-peptide hybrids. For example, oligonucleotides have been linked to a number of peptides to enhance the cellular membrane permeability [2], generate specific cleavage of nucleic acids [3] and provide attachment sites for non-radioactive reporter groups [4].

A number of methods of peptide-oligonucleotide conjugation have been described recently, none of which is sufficiently general or convenient. Two chemical schemes have mainly been adopted. In the first one, conjugation of different peptides to modulate the function of oligonucleotides often requires introducing a suitable tether containing the reactive group (e.g. -SH or -NH_2) to the oligonucleotide and post-synthetically adding the peptide as an active intermediate, followed by carrying out the coupling reaction in aqueous medium [5,6]. This includes utility of fully deprotected and purified species. Although this method is widely used, it suffers from some disadvantages, such as poor coupling efficiencies and several steps needed for the purification of the conjugate [7].

An alternative approach is to carry out the conjugate synthesis in a linear fashion on a single solid-phase support [8,9]. The difficulty is partly the need to find a combination of compatible protecting groups for the oligonucleotide and peptide moieties during sequential assemblies. All these drawbacks restrict the choice of conjugates that can be prepared.

In this paper, we describe a fragment strategy for the preparation of oligonucleotide-peptide conjugates that involves coupling of a N^{α}-protected peptide through its carboxy-terminus to the assembled solid support-bound oligonucleotide after generating the free amino group in the solid support, while other protecting groups are intact and oligonucleotide is still linked to the polymer support. A fragment coupling route was first described by Grandas and coworkers [10] for the preparation of peptide-oligonucleotide conjugates with a *N*-acylphosphoramidite union. Peyrottes et al. [11] have recently demonstrated the use of a fragment route for the coupling of peptide fragments to support-bound protected oligonucleotide through the formation of an amide linkage. This methodology takes advantage of mild reaction conditions which are convenient for the preparation of hybrid molecules. The utilization of these methods, however, is limited because peptides were conjugated at the 5′-terminus of a protected oligonucleotide. Furthermore, a more flexible approach may be needed where a peptide can be attached at any site along an oligonucleotide chain, since the site of conjugation may significantly influence the cellular uptake properties. Our general idea is based upon synthesizing a suitably protected nucleoside bearing a side arm which is attached to the 2′-position of a carbohydrate residue on one end and terminates in a masked primary amino group. Once synthesized, a conveniently prepared phosphoramidite derivative replaces one of the standard monomers in a solid-phase synthetic procedure. The advantage of this approach is that 'linker arm' nucleosides can be incorporated into the DNA at precisely defined sites and the subsequent binding of the peptide occurs only at those sites.

2. Materials and methods

2.1. General procedures

2-Cyanoethyl-*N,N*-diisopropyl phosphoramidites and controlled pore glass supports derivatized with protected deoxynucleosides were purchased from Applied Biosystems. 1*H*-tetrazole was purchased from Pharmacia. *N*-hydroxysuccinimide, *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU) and *N*-(9-fluorenylmethoxycarbonyloxy)succinimide were obtained from Aldrich. Dicyclohexylcarbodiimide (DCC) was purchased from Sigma. Anhydrous solvents were obtained using standard techniques.

^{1}H-NMR spectra were recorded on a VXR-400 spectrometer (Varian). Chemical shifts (δ) are expressed in parts per million from tetra-

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PII: S0014-5793(99)00921-7

methylsilane. Electrospray mass spectra were obtained using a Finnigan MAT LCQ spectrometer. MALDI mass analyses were performed on a Voyager DE spectrometer (PerSeptive Biosystems).

2.2. Preparation of modified nucleoside 3′-phosphoramidite

9-(2-Amino-2-deoxy-5-*O*-dimethoxytrityl-β-D-arabinofuranosyl)-N^6-benzoyladenine **1** was prepared from adenosine as described earlier [12]. *N*-(9-Fluorenylmethoxycarbonyl)-β-alanine and *N*-(9-fluorenylmethoxycarbonyl)-β-alanine *N*-hydroxysuccinimide ester **2** were synthesized according to literature procedures [13,14].

9-(2-Deoxy-2-(*N*-(9-fluorenylmethoxycarbonyl)-3-aminopropionyl)-amino-5-*O*-dimethoxytrityl-β-D-arabinofuranosyl)-N^6-benzoyladenine **3**. Compound **1** (0.67 g, 1 mmol) was dissolved in dry dioxane (10 ml) and *N*-(9-fluorenylmethoxycarbonyl)-β-alanine *N*-hydroxysuccinimide ester **2** (0.42 g, 1 mmol) was added on stirring. The reaction was then stirred for 8 h at room temperature. After removal of solvent and purification by column chromatography on silica gel, compound **3** was obtained (0.82 g, 85%). ^{1}H-NMR ($CDCl_3$, δ): 8.56 (s, 1H, H-8), 8.22 (s, 1H, H-2), 7.97–6.75 (m, 26H, Fmoc, 4′,4″-dimethoxytrityl, N^6-benzoyl), 6.44 (d, 1H, H-1′, $J_{1',2'}$ 5.9 Hz), 4.75 (m, 1H, H-2′), 4.40–4.11 (m, 4H, H-3′, H-9 of Fmoc and Fmoc-CH_2), 3.80 (m, 1H, H-4′), 3.55 (m, 2H, H-5′a,b), 3.05 (m, 2H, CH_2β of β-Ala), 2.50 (t, 2H, CH_2α of β-Ala, $J_{CH2\alpha,CH2\beta}$ 6.7 Hz). ESI mass spectrometry (MS): calculated for $C_{56}H_{51}N_7O_9$ 966.06. Found: *m/z* 966.1 (M^+), 967.1 $(M+H)^+$.

Phosphitylation of **3** was carried out according to Barone et al. [15]. Yield of **4**: 95%. ESI MS: calculated for $C_{65}H_{68}N_9O_{10}P$ 1166.28. Found: *m/z* 1166.3 (M^+). ^{31}P-NMR ($CDCl_3$, δ): −148.86, −149.18.

2.3. Oligodeoxynucleotide synthesis and characterization

Oligodeoxynucleotides were assembled using an Applied Biosystems 380B DNA synthesizer following the manufacturer's recommendations with the cyanoethyl phosphoramidite procedure. For couplings with modified phosphoramidite **4**, a 0.15 M concentration in anhydrous acetonitrile was used and the coupling wait time was increased to 600 s.

The oligonucleotides were deprotected, purified and analyzed by reversed phase high performance liquid chromatography (HPLC) with no modifications to the normal procedures [12].

2.4. Peptide synthesis and characterization

Synthesis of peptide fragments was carried out by Fmoc solution-phase chemistry. Peptide couplings employed N^α-Fmoc-protected amino acid building blocks, dicyclohexylcarbodiimide and hydroxybenzotriazole (HOBT) in DMF. Fmoc peptides were analyzed and isolated as described earlier [16].

2.5. Coupling of peptide fragments to oligodeoxynucleotides

The Fmoc protecting group was first removed from the support-bound oligodeoxynucleotide by treatment with morpholine for 1 h at room temperature. The supernatant was then decanted and support was rinsed with DMF (2×100 μl). Finally, to the oligodeoxynucleotide-resin were added: Fmoc peptide (five equivalents compared to oligodeoxynucleotide), HBTU (one equivalent compared to peptide) and *N,N*-diisopropylethylamine (10 equivalents) in 100 μl DMF. The solution was then shaken from time to time for 1.5 h at room temperature. Following removal of supernatant, the solid support was washed with DMF (3×100 μl) and then with ethanol (2×100 μl). Cleavage from polymer support, deprotection, analytical and preparative HPLC of conjugates were carried out as mentioned above for oligonucleotides. Conjugate molecular masses were determined by MALDI-TOF MS (Table 1).

3. Results and discussion

Our initial studies have concentrated on preparation of a modified nucleoside building block in which linker is incorporated for attachment of the peptide fragment. A spacer arm of some length is desirable to separate the inserted functionality from the nucleic acid sequence, especially if the oligomer is to be hybridized to a complementary target. The ideal location for conjugation of different ligands is at sites which have steric tolerance in a double helix. The 2′-position of a sugar ring is not involved in hydrogen bonding so the attachment of a linker molecule or peptide fragment should not drastically alter the stability of the nucleic acid complex. For this reason, and because of simple synthesis procedures being used in its preparation, amine linker arm nucleoside was accepted by us.

We recently reported [12] the synthesis of the new type of sugar-modified oligodeoxynucleotides containing 9-(2-amino-2-deoxy-β-D-arabinofuranosyl)adenine residues. The 2′-amino-2′-deoxyarabinoadenosine residues were shown to induce the increased resistance of modified oligomers towards the enzymatic cleavage and provide insignificant destabilization of DNA duplexes. Therefore, the functionalization of oligonucleotides by incorporating 2′-aminonucleoside may provide a way to link various groups to the carbohydrate moiety. A 2′-amido bond may be expected to be chemically and metabolically stable and not interfering with oligonucleotide synthesis. Thus 2′-amino-2′-deoxyarabinoadenosine was selected as precursor of amino linker containing nucleoside.

The length of the linker molecule is very critical for a specific use. Using our previous expertise in synthesis of cross-linked DNA duplexes [17], β-alanine was proposed to incorporate an amino group at the 2′-position. Preparation of the linker molecule requires appropriate protection of the amino group. It must be chemically blocked but specifically removable without affecting the remaining protecting group on the oligonucleotide or cleaving the linkage to the solid-phase. In order to insert amino linker containing nucleosides at any position of oligonucleotides which can be applied for solid-phase conjugation chemistry, we introduced a Fmoc protecting group. The Fmoc group is ideal for protection of primary amines during oligonucleotide synthesis since it is stable to hydrolysis in the organic acids used to remove 5′-*O*-dimethoxytrityl groups in each cycle, yet, it undergoes rapid non-

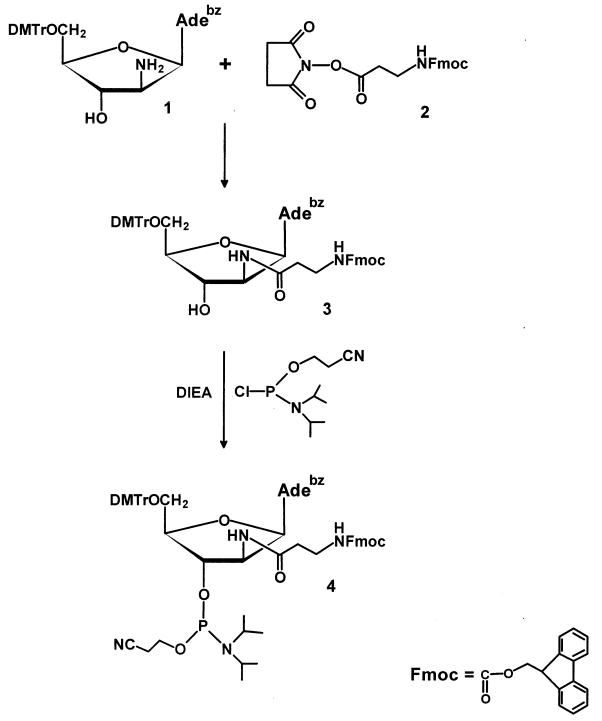

Fig. 1. Synthesis of the phosphoramidite derivative of 2′-amino linker containing nucleoside analogue.

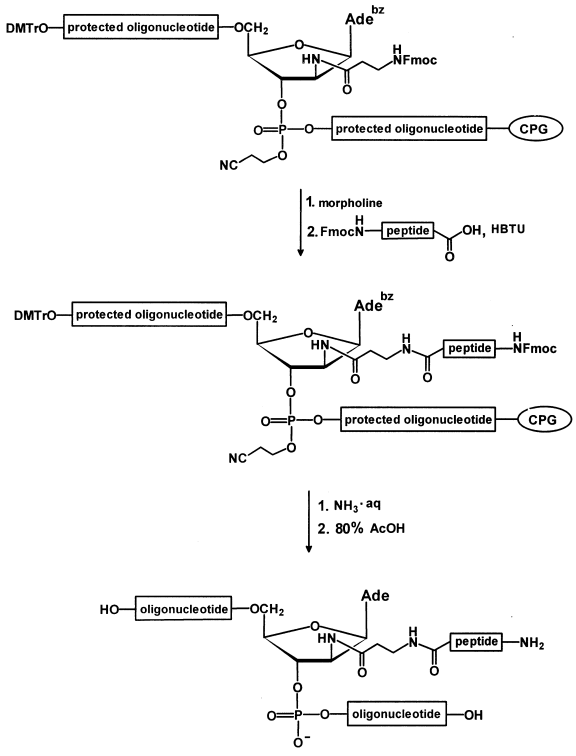

Fig. 2. Reaction scheme for the preparation of oligonucleotide-peptide conjugates.

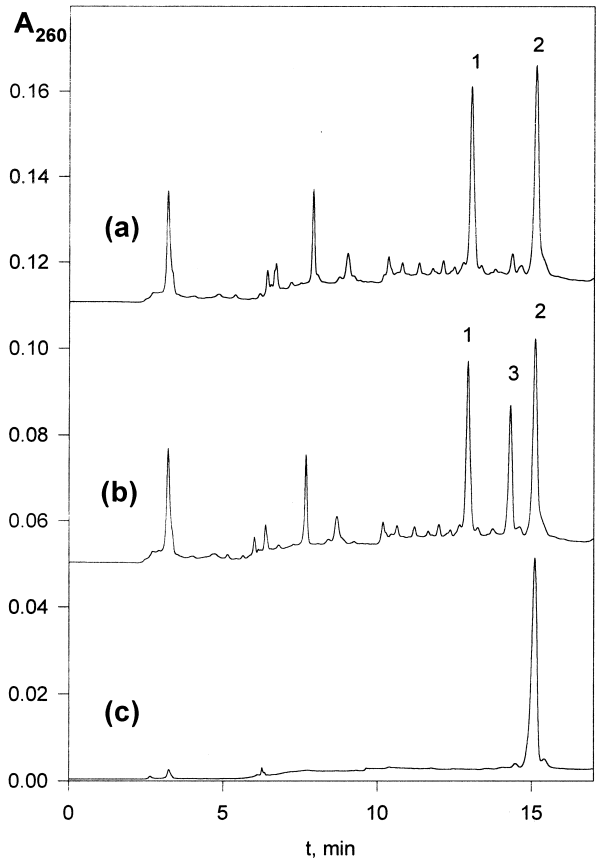

Fig. 3. HPLC profiles of (a) the reaction mixture resulting from solid-phase coupling of N^{α}-Fmoc-Leu-Gly to support-bound 12-mer (**II**), (b) the reaction mixture (as shown in a) co-injected with purified 12-mer (**II**), (c) purified oligonucleotide-peptide conjugate (c1, Table 1). For conditions, see Section 2. Peak 1, 5′-CCGCGCT-3′ (the by-product, failure sequence); peak 2, c1; peak 3, 5′-GACCA*CCGCGCT-3′.

hydrolytic cleavage on treatment with simple bases (piperidine, morpholine), usually in polar solvents such as DMF or acetonitrile, to liberate an amino group in the free base form. Therefore, we envisioned that these conditions would be mild enough to preclude removal of β-cyanoethyl protecting groups from internal phosphates or damaging the linkage between the solid support and the oligonucleotide. Prior to the synthesis of oligodeoxynucleotide chains, the stability of the standard succinyl linkage to the morpholine was analyzed. After the assembly of the dinucleoside phosphate TT, the support was treated with morpholine. In this case, the succinyl linkage was found to be stable under these conditions (HPLC analysis). We also observed a good stability of the β-cyanoethyl protecting group when morpholine was added to tritylated dimer TT synthesized by the phosphoramidite method in solution [18] (TLC analysis).

Synthesis of 2′-deoxyadenosine analogue bearing amino linker was carried out as shown in Fig. 1. Compound **1** was made starting from adenosine as previously described [12]. Thus, reaction of β-alanine with *N*-(9-fluorenylmethoxycarbonyloxy)succinimide in dioxan/water (1:1) afforded a 95% yield of *N*-(9-fluorenylmethoxycarbonyl)-β-alanine. The product reacted with *N*-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide in tetrahydrofuran solution to give the corresponding succinimidyl derivative **2** in a 75% yield. The 2′-amino group of the appropriately blocked nucleoside **1** reacted selectively with **2** to provide functionalized linker arm nucleoside analogue **3** which was converted to its 3′-phosphoramidite **4** by standard methods [15].

Upon completion of each oligonucleotide synthesis, the 5′-*O*-dimethoxytrityl group was left on to aid reversed phase HPLC purification. The feasibility of the synthesis was first tested by taking a heptamer, 5′-TTTT**A***TT-3′ (**I**) (**A***, 9-(2-(3-aminopropionyl)amino-2-deoxy-β-D-arabinofuranosyl)adenine), as a model sequence. The strategy was extended to the 12-mer 5′-GACC**A***CCGCGCT-3′ (**II**), complementary to part of the 5S RNA from *Escherichia coli*. When modified phosphoramidite synthon **4** was used, standard procedures for synthesis with normal ones had to be altered by extending

Table 1
MS characterization of oligonucleotide-peptide conjugates and percentage yields in the coupling reactions

Oligonucleotide/peptide	5′-GACC**A***CCGCGCT-3′				
	Abbreviations	Empirical formula	Expected	*m/z* found	Yield[a], %
Leu-Gly	c1	$C_{125}H_{166}N_{49}O_{72}P_{11}$	3 847.68	3 846.88	90.3
Tyr-D-Ala-Phe-Gly	c2	$C_{140}H_{178}N_{51}O_{75}P_{11}$	4 115.96	4 114.01	81

[a]Yields are based on HPLC analysis.

the coupling time to 10 min. As a further check on the synthetic procedure, one batch of either synthesis was deprotected by standard protocols and analyzed by equidistant ion-pair reversed phase HPLC [19]. After synthesis, an aliquot of **II** was deprotected and purified by reversed phase HPLC. A major component corresponding to the desired product was observed (data not shown) and characterized by MALDI-TOF MS.

In order to certify the existence of the amino group, 7- and 12-mers were treated with acetic anhydride to give acetylated oligonucleotides, retention times of which were somewhat longer than that of corresponding oligomers with a free amino group (data not shown).

The blocked oligodeoxynucleotide attached to the controlled pore glass support was deprotected to unmask the tethered amino group in the solid support (Fig. 2). In this case, the Fmoc protecting group on the β-alanine residue was removed by treatment with morpholine for 1 h.

To illustrate the solid-phase synthesis of oligonucleotide-peptide conjugates, we chose two short peptides, N^{α}-Fmoc-Leu-Gly and N^{α}-Fmoc-Tyr-D-Ala-Phe-Gly, that contain a C-terminal glycine, where racemization cannot take place. Peptide fragments were prepared using standard DCC/HOBT-mediated couplings [20] of *N*-Fmoc-protected α-amino acids in solution. During the past two decades, a number of different reagents have been used for amide bond formation. Of these, activator HBTU is one of the best reagents for in situ formation of hydroxybenzotriazolyl esters [21]. It enables couplings to be carried out smoothly and the free aliphatic hydroxyl function of tyrosine is not affected. In our procedure, the support-bound oligodeoxynucleotide reacted with a solution of Fmoc peptide in dimethylformamide in the presence of HBTU and triethylamine for 1.5 h. An additional advantage of our process is the facile purification of the final product containing a terminal 5′-*O*-dimethoxytrityl group by reversed phase HPLC. The conjugate molecule was analyzed by equidistant ion-pair reversed phase HPLC and by MALDI-TOF MS (Table 1). For coupling reactions to the support-bound amino-functionalized oligonucleotide (**II**), good yields were obtained for both dipeptide Leu-Gly and tetrapeptide Tyr-D-Ala-Phe-Gly conjugates. An example is shown in Fig. 3. It was observed that the major peak corresponding to the peptide conjugate of oligonucleotide (peak 2) was retarded in mobility compared to control oligonucleotide functionalized with a primary amino group at its 2′-position (peak 3).

To sum up, we developed a new flexible strategy where protected peptide fragments were coupled to solid-phase-bound, fully protected oligodeoxynucleotides to form irreversible covalent linkages. The formation of a stable amide bond was achieved by conjugation of the C-terminus of the peptide to the 2′-amino-functionalized oligonucleotide. The 2′-functionalization methodology proved to be extremely valuable for preparing two peptide conjugates of oligodeoxynucleotides. Once high yields have been obtained for short peptide conjugations, it will be possible to attach longer peptides. The studies will be considerably extended to include peptide conjugates of oligonucleotide analogues that are likely to be useful as antisense agents.

Acknowledgements: Authors sincerely thank Dr Dmitry A. Stetsenko for MALDI MS analysis, Antonina N. Muravyeva for performing the reversed phase HPLC and Anna V. Kachalova for consulting on ^{1}H-NMR. This work was supported by funds from the Russian Foundation for Basic Research (Grant 97-0448624) and Universities of Russia, fundamental research program (Grant 5-5166).

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