Synthesis of Peptide−Oligonucleotide Conjugates with Single and Multiple Peptides Attached to 2′-Aldehydes through Thiazolidine, Oxime, and Hydrazine Linkages

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2′-Deoxyoligonucleotides and 2′-*O*-methyloligoribonucleotides carrying one or more 2′-aldehyde groups were synthesized and coupled to peptides containing an N-terminal cysteine, aminooxy, or hydrazide group to give peptide−oligonucleotide conjugates incorporating single or multiple peptides in good yield. The facile conjugation method allows specific coupling in aqueous solution of unprotected oligonucleotides containing aldehyde groups to unprotected N-terminally modified peptides and other small molecules. A 12-mer 2′-*O*-methyloligoribonucleotide complementary to the HIV-1 TAR RNA stem-loop and containing two conjugated copies of an 8-mer model laminin peptide was hardly affected in TAR RNA binding and showed a similar level of inhibition of HIV-1 Tat-dependent in vitro transcription compared to the unconjugated 2′-*O*-methyloligoribonucleotide. Advantages of this conjugation method include (1) the ability to attach more than one peptide or other small molecule to oligonucleotide at defined nucleoside residue locations; (2) a conjugation route that does not affect significantly oligonucleotide binding to RNA structures; and (3) three alternative, facile, and mild conjugation reaction types that do not require use of a large excess of peptide reagent.

INTRODUCTION

Oligonucleotides and their analogues have been studied for more than two decades as specific inhibitors of gene expression. Oligonucleotides can be used to target mRNA by antisense (*1*) or RNAi (*2*) mechanisms, double-stranded DNA by triplex formation (*3*), or proteins by aptamer selection (*4*). Oligonucleotides conjugated to certain peptides have been found to possess improved cell-specific targeting, cellular uptake efficiency, or stability to degradation in comparison to unmodified oligonucleotides (*5*−*7*). In some cases improved binding strength to RNA and target specificity has been achieved (*8*).

Several methods of chemical synthesis of peptide−oligonucleotide conjugates have been developed to date (*5*). The two most popular synthetic approaches are total stepwise solid-phase synthesis and coupling in solution of separately prepared peptide and oligonucleotide fragments. In total synthesis, the same solid support is used for both peptide and oligonucleotide assemblies. Peptide synthesis may be followed by oligonucleotide synthesis (*9*), or vice versa (*10*), or a branched linker is attached to the support permitting independent growth of both peptide and oligonucleotide chains (*11*). Unfortunately, the chemistries of peptide and oligonucleotide synthesis have been poorly compatible up to now. Attempts to solve these problems include modification of the solid support (*12*), base protecting groups of nucleoside components (*13*), side-chain amino acid building blocks (*14*), or deprotection conditions (*15*). Some recent advances in total solid-phase synthesis look promising (*16*), but so far no routine procedure has emerged.

Fragment coupling of peptides to oligonucleotides has found particular utility in the case of longer peptides. One method involves coupling of a protected peptide segment to a support-bound, protected, or partially unprotected oligonucleotide (*17*). A particular advantage of this method is that, as for regular solid-phase synthesis, the excess of reagents is removed easily by filtration. However, coupling yields have been found to be poor in the case of long or "difficult" peptides. A more general approach is the separate solid-phase assembly of oligonucleotide and peptide fragments, deprotection (and purification if necessary) followed by a solution-phase chemoselective ligation mediated by mutually reactive groups introduced into each component during solid-phase assembly or postsynthetically. These methods include formation of disulfide (*18*), thioether (*19*), amide (*20*), oxime (*21*, *22*), thiazolidine (*22*), or imine (*23*) linkages.

An alternative route to synthesis of peptide−oligonucleotide conjugates is by an addition−elimination reaction of a nucleophile to an electrophilic aldehyde group. Here, weakly basic nucleophiles attached to the N-terminus of a peptide should be suitable because they would be expected to have a pK_a lower than that of peptide side chain amino groups (such as of Lys and Arg) and thus should react with aldehydes at pH near or below 7, where aliphatic amino groups of peptide should be

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10.1021/bc020016+ CCC: $22.00
Published on Web 06/25/2002

largely protonated. The use of unprotected peptide fragments has several advantages. First, unprotected peptides are usually water-soluble or can be solubilized by addition of organic cosolvents, such as DMF, or denaturing agents, such as urea or guanidinium chloride. Second, products of conjugation reactions may be readily purified by PAGE or HPLC, do not require additional deprotection steps, and thus may be used directly for biological assays. Appropriate nucleophiles which may react with aldehyde groups include *O*-alkyl hydroxylamines, arylhydrazines, acyl- and sulfonylhydrazides, and carbazates. The linkages thus formed are correspondingly oxime and hydrazone, which are sufficiently stable, yet are prone to cleavage by hydrolysis under forcing conditions. Further, 1,2-aminothiols react with carbonyl groups to form thiazolidines, which are also stable over a wide pH range (*24*). Normally, such conjugation reactions are conducted at a slightly acidic pH.

The advantages of an aldehyde group include the possibility of a reversible additive-free conjugation with a range of nucleophiles without an additional preactivation step that is required for other carboxylic groups. There are two well-known ways to introduce an aldehyde group into oligonucleotides through use of solid-phase phosphoramidite chemistry. One route involves use of a masked aldehyde moiety (*25*) such as an acetal. A second approach involves use of a precursor incorporating a suitably protected 1,2-diol group (*26*). Here an aldehyde can be generated under the more convenient and mild conditions of periodate oxidation (*26*), whereas demasking conditions of an aldehyde group can sometimes be harsh (*25*). 1,2-Diols can be obtained by OsO_4-catalyzed hydroxylation of alkenes (*27*). We described recently a new phosphoramidite reagent for incorporation of 1,2-diol groups into oligonucleotides using 2′-modification (*28*). Conjugation of the subsequently generated 2′-aldehydes may be effected through use of any of three alternative chemistries and thus provides the possibility of introducing multiple peptides into defined positions within an oligonucleotide sequence.

The formation of thiazolidines using N-terminal cysteinyl peptides has been used widely for protein ligation and peptide dendrimer formation (*29*). Cysteine residues can also be introduced at the N-terminus of oligonucleotides by use of phosphoramidite reagents developed recently (*30, 31*). A cysteinyl group has been used frequently for peptide–oligonucleotide conjugation through disulfide or thioether formation (*5*), or more recently for native ligation of peptide (*30*) or oligonucleotide thioesters (*32*). Use of organic cosolvents and elevated temperature leads to consistently better results than aqueous buffer alone (*33*). However, the most common polar aprotic solvents DMF and DMSO are not suitable in the case of thiazolidine formation. DMF use results in side reactions, e.g., formylation of peptide amino groups, and DMSO is a mild oxidizing agent that can cause disulfide formation of cysteine peptides (*29*). Use of NMP or acetonitrile as cosolvents usually gives better results.

An *O*-alkyl hydroxylamino group, more specifically an aminooxyacetyl group, has been widely used for peptide ligation (*29, 34, 35*). Recently aminooxy groups have been incorporated into oligonucleotides (*21*) and used in conjugation reactions with reporter groups (*36, 37*) or modified peptides (*21, 22*). Not only is an oxime linkage stable over a wide pH range (*33*), but aminooxyacetic acid per se or Boc-protected is commercially available. The latter can be used directly in the final coupling step in solid-phase peptide synthesis.

Hydrazone formation seems to be a favorite method for coupling of various groups in carbohydrate and protein chemistry (*38*). Sometimes, a reduction by $NaBH_3CN$ is needed to increase the stability of such conjugates to acidic or basic treatment. Peptide hydrazides can be easily generated from esters by hydrazinolysis. An alternative approach that is better suited for solid-phase synthesis is to introduce Fmoc-4-hydrazinobenzoic acid on to the N-terminus of a peptide (*29*). However, since this method seemed not to be altogether successful, we have used our previously developed thiosuccinate reagent, pentafluorophenyl *S*-benzylthiosuccinate (*30*), to generate a succinic acid monohydrazide on the N-terminus of a peptide after a mild hydrazine treatment.

A few papers describe the synthesis of peptide–oligonucleotide conjugates where a peptide is attached to the 2′-position via a 2′-aminonucleoside (*39–41*), but this route can suffer from low yields (*40, 41*). Further, the incorporation of 2′-aminonucleosides and their acyl derivatives strongly decreases duplex stability (*42*).

2′-*O*-Alkyloligonucleotides are widely used in antisense research. As described in a recent review (*43*), 2′-*O*-alkyloligonucleotides in general show improved binding affinity to target mRNA, a large increase in nuclease resistance, and added stability against depurination compared to unmodified oligodeoxyribonucleotides. Incorporation of multiple 2′-*O*-alkylnucleotides is known to preserve an A-type duplex structure when bound to an RNA strand, since, in contrast to 2′-aminonucleosides, 2′-*O*-alkyl-modified nucleosides adopt a sugar conformation similar to that of ribonucleotides. Pendant peptides conjugated through a 2′-*O*-alkyl modification would be expected to be positioned on the minor groove side of an oligonucleotide–RNA duplex. Considering these potential benefits for peptide–oligonucleotide conjugates, we now report the highly efficient conjugation of peptides, either singly or multiply through 2′-aldehydes, to oligodeoxynucleotides and to 2′-*O*-methyloligoribonucleotides. Further, we show that such conjugates are not impaired significantly in their ability to bind to complementary RNA, and in some cases binding is enhanced. We also report that a 12-mer 2′-*O*-methyloligoribonucleotide complementary to the HIV-1 TAR RNA stem-loop and containing two conjugated copies of an 8-mer model laminin peptide was able to inhibit HIV-1 Tat-dependent in vitro transcription as well as the umodified 2′-*O*-methyloligoribonucleotide, suggesting that such conjugation routes should be suitable in principle for attachment of single or multiple cell-penetrating or cell-targeting peptides.

EXPERIMENTAL SECTION

General. Materials were obtained from commercial suppliers and used without further purification unless otherwise noted. Polystyrylmethyl trimethylammonium metaperiodate (loading 2.17 mmol/g) and polystyrylmethyl trimethylammonium borohydride (loading 3.65 mmol/g) were purchased from Novabiochem, creatine phosphate, creatine kinase, poly[d(I-C)] (Boehringer Mannheim), RNAsin, transcription plasmid phRL-CMV (Promega). Analytical HPLC of peptides was carried out with dual wavelength (215 and 230 nm) UV detection using a Vydac RP-C8 column (4.6 × 250 mm). Preparative peptide HPLC was carried out using a Vydac RP-C8 column (25 × 300 mm) and 215 nm UV detection. A gradient of acetonitrile in 0.1% aq TFA from 10 to 90%

in 30 min was used for the HPLC analysis and purification of peptides. HPLC analysis and purification of 5′-O-Dmt-protected oligonucleotides and conjugates were carried out on a Tracor instrument using 4 × 250 mm DIAKS-130-CETYL column; buffer A: 0.1 M ammonium acetate (pH 7); buffer B: 0.1 M ammonium acetate, 40% MeCN, (pH 7); gradient of B from 0 to 100% in 60 min; flow rate 1 mL/min; temperature 45 °C. Oligonucleotides and conjugates were analyzed by a reversed phase HPLC (ion-pair mode) (Waters) on a DIAKS-130-CETYL column (4 × 250 mm) using a logarithmic gradient: 0−45.9% B (1 min); 45.9−49.2% B (1 min); 49.2−53.6% B (3 min); 53.6−56.9% B (5 min); 56.9−60.2 B (10 min); 60.2−62.1% B (10 min); 62.1−63.5% B (10 min). Separation of oligomers with a retention time step of 1 min/unit was carried out; mobile phase A: H_2O-MeCN (95:5 v/v), 2 mM tetrabutylammonium dihydrogen phosphate, 48 mM KH_2PO_4, pH 7; mobile phase B: H_2O-MeCN (60:40 v/v), 2 mM tetrabutylammonium dihydrogen phosphate, 48 mM KH_2PO_4, pH 7; flow rate 1 mL/min, and temperature 45 °C. MALDI-TOF mass spectra were run on a Voyager DE workstation (PE Biosystems) in a freshly prepared 1:1 v/v mixture of 2,6-dihydroxyacetophenone (40 mg/mL in MeOH), and aq diammonium hydrogen citrate (80 mg/mL) as a matrix. Denaturing gel electrophoresis of oligonucleotides was performed in 15% PAGE containing 2 M urea in Tris-borate buffer (50 mM Tris HCl, 50 mM boric acid, 1 mM EDTA, pH 7.5).

Peptide Synthesis and Characterization. Peptides **6** and **7** were purchased from Bachem. Solid-phase peptide synthesis of peptides **8** and **9** was carried out by the solid-phase Fmoc/*tert*-butyl procedure (*44*) on a PE Biosystems Pioneer peptide synthesizer on a 0.05 mmol scale using HATU/DIEA in situ activation protocol (*45*) and Rink amide NovaGel HL support (Novabiochem). Boc-Aminooxyacetic acid for **8** and *S*-benzyl thiosuccinic acid (*30*) for **9** were placed at the last amino acid position and incorporated at the end of normal Fmoc-on solid-phase synthesis protocol by a standard coupling cycle. In the case of **9**, the resin was removed from a column, treated with 0.5 M hydrazine hydrate solution in dioxane for 2 h at room temperature, filtered, washed by DMF, MeOH, and diethyl ether, and finally dried in vacuo. Both peptides **8** and **9** were subjected to normal acidolytic cleavage and deprotection procedure by TFA−phenol−1,2-ethanedithiol−triisopropylsilane−water (900:25:25:25:25 v/v/v/v). Resulting peptides were analyzed by RP-HPLC, their respective molecular masses confirmed by a MALDI-TOF analysis; matrix: 10 mg/mL α-cyano-4-hydroxycinnamic acid, 3% (v/v) TFA in H_2O/MeCN (1:2 v/v).

Oligonucleotide Synthesis and Characterization. Oligodeoxyribonucleotides and 2′-*O*-methyloligonucleotides were assembled on a ABI 380B DNA synthesizer by the cyanoethyl phosphoramidite method (*46*) following manufacturer's recommendations. Protected 2′-deoxy- and 2′-*O*-methylnucleoside phosphoramidites were purchased from Glen Research. Prepacked 0.4 μmol columns were used throughout. For couplings with modified phosphoramidite (*28*), 0.15 M concentration in anhydrous acetonitrile was used, and the coupling time was increased to 15 min. Oligonucleotides were cleaved from the support and deprotected using concentrated aq ammonia overnight at 55 °C.

Synthesis of 2′-Aldehyde Oligonucleotides. *Method A (for subsequent thiazolidine conjugation).* To a solution of 2′-diol oligonucleotide (5 A_{260} units, 43 nmol) in 50 mL of 0.2 M acetate buffer, pH 4.5, were added a few granules of polystyrylmethyl trimethylammonium metaperiodate. After 1 h of shaking, the solution was decanted and polymer washed by H_2O (2 × 20 mL). The solution was evaporated in vacuo to remove volatiles.

Method B (other cases). To a solution of 2′-diol oligonucleotide (5 A_{260} units, 43 nmol) in 50 mL of 0.2 M acetate buffer, pH 4.5, was added 5 mL of 0.1 M $NaIO_4$. After 30 min, the reaction mixture was evaporated in vacuo.

Coupling of N-Terminal Cysteine Peptides to 2′-Aldehyde Oligonucleotide. A solution of peptide **6** or **7** (50−70 nmol) in a mixture of 0.4 M acetate buffer, pH 4.5, and acetonitrile (3:7−7:3 v/v, 50 mL) was added to the oligonucleotide **I**. The reaction mixture was incubated at 37 °C for 0.3−18 h and then evaporated in vacuo and purified by PAGE.

Coupling of *O*-Alkylhydroxylamine Derivatives to 2′-Aldehyde Oligonucleotides. A solution of *O*-alkylhydroxylamine derivative (100 nmol of **2** and **5** or 60−70 nmol of **8**) in 50 mL of the mixture of 0.4 M acetate buffer, pH 4.7, and DMSO (3:7−7:3 v/v) was added to an oligonucleotide. The reaction mixture was incubated at 37 °C for 0.3−5 h, depending on the molecular weight of the conjugated molecule and purified by PAGE in the case of peptide conjugates. Other conjugates were precipitated by addition of 0.1 mL of 2 M $LiClO_4$ and 1 mL of acetone and purified by RP-HPLC.

Coupling of Hydrazines and Hydrazides to 2′-Aldehyde Oligonucleotides. A solution of hydrazine or hydrazide (100 nmol of **3** or 60−70 nmol of **9**) in 50 mL of the mixture of 0.4 M acetate buffer, pH 4.7, and DMSO (3:7−7:3 v/v) was added to an oligonucleotide. The reaction mixture was incubated at 37 °C for 0.3−5 h, and then a few granules of polystyrylmethyl trimethylammonium borohydride were added. The reaction mixture was incubated for 30 min more and purified by PAGE in the case of **9**. In the case of **3**, the conjugate was precipitated by addition of 0.1 mL of 2 M $LiClO_4$ and 1 mL of acetone and purified by RP-HPLC.

Analysis of Hydrolytical Stability of Thiazolidine and Oxime Conjugates. Conjugates (0.2 A_{260} units) were dissolved in 20 mL of 0.2 M acetate buffer, pH 2.0, 3.0, or 4.0, or 0.2 M phosphate buffer, pH 8.0, 9.0, or 10.0. After 24 h, conjugates were precipitated by addition of 0.1 mL of 2 M $LiClO_4$ and 1 mL of acetone.

Analysis and Characterization of Peptide−Oligonucleotide Conjugates. Product elution from gel was performed with 0.5 M aq sodium acetate for 12 h at 4 °C. Conjugates were precipitated by addition of 1 mL of EtOH followed by cooling at −70 °C for 1 h. RP-HPLC (ion-pair mode) analyses were carried out as described previously (*28*). Purified conjugates were then analyzed by MALDI-TOF mass spectrometry.

Thermal Melting Experiments. Absorbance vs temperature profiles were measured on a Varian Cary 50 spectrophotometer in a buffer containing 100 mM NaCl, 10 mM Na_2HPO_4, 1 mM EDTA, pH 7.0. Oligonucleotides, conjugates, and their complementary templates were combined at 4 μM of each strand, heated at 90 °C, and cooled at a rate of 0.5 °C/min in a 1 cm path length cell and then heated back to confirm reversibility and lack of evaporation.

In Vitro Transcription Inhibition. Cell-free transcription reactions with DNA template derived from plasmid p10SLT carrying the wild-type HIV-1 LTR (strain NL4-3) were carried out as described previously (*47*, *54*). Briefly, the reaction mixtures (40 μL) containing 15 μL of HeLa cell nuclear extract (*48*), 10 nM −345 to +524 fragment (*Eco*RV-Xba I) of plasmid p10SLT, 80 mM KCl, 3.5 mM $MgCl_2$, 20 mM HEPES (pH 7.9), 2 mM DTT,

10 μM $ZnSO_4$, 10 mM creatine phosphate, 100 μg mL^{-1} creatine kinase, 1 μg of poly[d(I-C)], 50 μM ATP, GTP, and CTP, 5 μM UTP, [α-^{32}P]-UTP (10 μCi), 1 unit mL^{-1} RNAsin, 200 ng of recombinant Tat protein (*48*), and increasing concentrations of inhibitor oligonucleotide were incubated at 30 °C for 20 min and then stopped by addition of 50 μL of 150 mM sodium acetate solution, 0.5% SDS, 10 mM EDTA, and 20 μg mL^{-1} tRNA. After phenol/chloroform extraction and ethanol precipitation, the reaction products were analyzed by 6% PAGE containing 7 M urea followed by autoradiography. The autoradiographs were scanned densitometrically using a Personal SI (Molecular Dynamics, Inc.), and the resulting digitized images were analyzed by Geltrak software (*49*).

For HIV Tat-independent in vitro transcription plasmid phRL-CMV was linearized using Nhe I. Transcription reactions were carried out essentially as in Tat-dependent transcription with minor differencies. The amount of DNA template was 200 ng, the concentration of KCl was decreased to 50 mM and the HIV Tat protein was not added to the reaction mixture. Tat-independent transcription inhibition was assessed at 0.5 and 2 μM concentrations of the inhibitor.

RESULTS AND DISCUSSION

We have described recently the introduction into oligonucleotides of 2′-*O*-(2,3-dihydroxypropyl)uridine as a suitable precursor of an aldehyde group (*28, 50*). The hydroxyl functions of the 1,2-diol in the modified residue were protected by benzoyl groups, which are removable during standard oligonucleotide deprotection with ammonia solution (Scheme 1). For peptide conjugation studies, we chose to introduce 2′-diol-modified uridine in place of thymidine or uridine residues within oligonucleotides complementary to the HIV-1 TAR RNA apical stem-loop, the target sequence for the HIV-1 trans-activator protein Tat (*51*). We have already shown that such oligonucleotides inhibit Tat binding to TAR RNA in vitro (*52, 53*) and that a 12-mer 2′-*O*-methyloligoribonucleotide inhibits Tat-dependent in vitro transcription (*54*). Both oligodeoxynucleotides (**I**, **II**, **III**, and **VII**) and 2′-*O*-methyloligoribonucleotides (**IV**, **V**, and **VI**) were synthesized containing diol modifications.

Sequence, 5′-3′:

CTCCCAGGCU̲CA **(I)**

CU̲CCCAGGCTCA **(II)**

CU̲CCCAGGCU̲CA **(III)**

cucccaggcU̲ca **(IV)**

cU̲cccaggcuca **(V)**

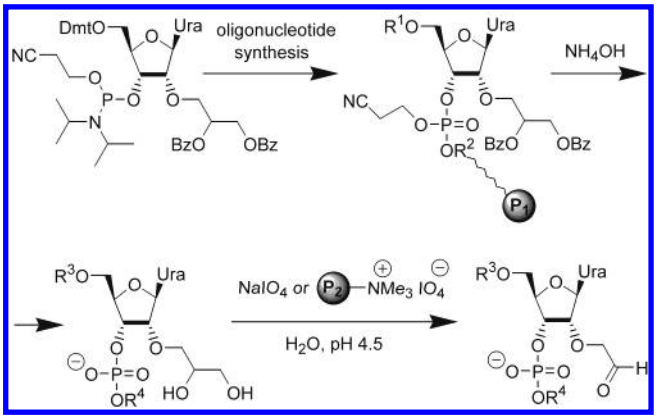
cU̲cccaggcU̲ca **(VI)**

GCU̲CCCAGGCTCAAA **(VII)**

where U̲ is 2′-*O*-(2,3-dihydroxypropyl)uridine, 2′-deoxynucleotides are capitalized, and 2′-*O*-methyl nucleotidesare in lower case

Oxidation of the 1,2-diol group was carried out under mild conditions, namely a 2-fold excess of $NaIO_4$ in acetate buffer, pH 4.5, 30 min. Polymer-supported periodate may also be used, in which case the reaction time was extended to 1 h (Scheme 1). The latter method is especially suitable for a subsequent thiazolidine conjugation, since it requires just a simple filtration step to remove the excess of periodate. The reaction is not very sensitive to pH, thus allowing a variety of buffers to be used. Subsequent conjugations can be carried out without purification of the aldehyde, except for thiol-containing compounds, which may be oxidized to disulfides by an excess of periodate.

Scheme 1[a]



[a] R^1, R^2 = protected oligonucleotide chain; R^3, R^4 = unprotected oligonucleotide chain; P_1 = controlled pore glass, P_2 = macroporous polystyrene.

For initial conjugation experiments (Scheme 2), we have chosen several model compounds and short peptides (Table 1). Peptide **6** is a glycoprotein hormone α (32−46) receptor binding domain (*55*). Peptide **7** incorporates SV-40 large T antigen NLS sequence (*56*). N-Terminally functionalized peptides **8** and **9** include residues 926−933 of laminin B1 chain. This sequence is known to bind laminin receptors and inhibits angiogenesis and tumor growth (*57*). We have studied the effects of pH, stoichiometry, organic solvent addition, and temperature on the reaction efficiency.

When the pH was increased from 3.5 to 6.0, the time of complete reaction (>95%) was also increased, in the case of L-cysteine ethyl ester from less than 5 min to 20 min. In the case of *O*-benzylhydroxylamine and 4-hydrazinobenzoic acid better results were obtained at pH 4.5−5.0, since after 3 h at pH 3.0 traces of degraded oligonucleotide were observed (data not shown). Therefore, thiazolidine formation was carried out at the safer pH 4.5. The effect of stoichiometry was less significant. Use of a 10-fold excess of nucleophile resulted in reaction proceeding twice as fast as when a nearly equimolar ratio was used. The reaction was therefore routinely carried out in the presence of 1.2−1.8 equiv of nucleophilic peptide.

As previously mentioned, DMF and DMSO are unsuitable for use during thiazolidine formation. Acetonitrile, which can be removed easily by evaporation, and NMP provided the best results, while formamide strongly decreased the efficiency of the reaction (data not shown). The best cosolvent for oxime and hydrazone conjugation was DMSO. At elevated temperature (37 °C) the efficacy of all reactions was increased also. In the case of hydrazinobenzoic acid, the resultant hydrazone was reduced to the corresponding hydrazine linkage by use of polystyryl trimethylammonium borohydride.

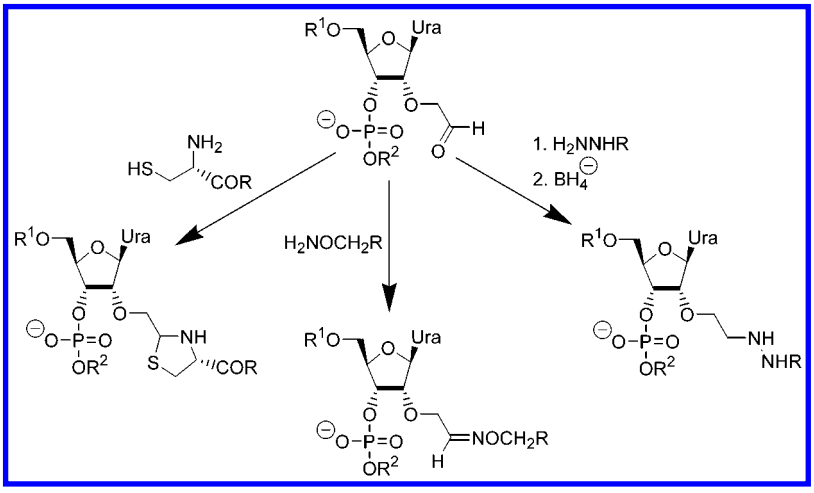
Conjugates of 2′-aldehyde-containing oligonucleotides with *O*-benzylhydroxylamine showed two peaks (Figure 1b, peaks *2* and 3) with close retention times on reversed-phase HPLC in ion-pair mode. After reduction by $NaBH_3CN$, only one peak (Figure 1c, peak 4) on the HPLC trace was observed. We therefore conclude that the two peaks represent oxime syn- and anti-isomers.

Table 1. Oligonucleotide 2′-Conjugates Synthesized, Yields of Crude Products and Isolated Yields (figure following oblique lines)

			oligonucleotide						
type[a]		conjugated molecule/peptide	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**
T	**1**	H-Cys-OEt	>90	>90	-	-	-	-	-
O	**2**	H_2NOCH_2Ph	>90	>90	-	-	-	-	90
H	**3**	$4\text{-}NH_2NHC_6H_4CO_2H$	85	85	70	-	-	-	-
T	**4**	H-Cys-Gly-OH	>90	>90	90	>90/70	90/65	90/70	-
O	**5**	$H_2NOCH_2CO_2H$	>90	>90	85	-	-	-	-
T	**6**	H-CFSRAYPTPLRSKKT-OH	90/35	-	-	-	-	-	-
T	**7**	H-CGYGPKKKRKVGG-OH	90/25	-	-	-	-	-	-
O	**8**	$H_2NOCH_2CO\text{-}DPGYIGSR\text{-}NH_2$	-	>90/65	90/45	>90/50	90/55	80/45	-
H	**9**	$H_2NNHCO(CH_2)_2CO\text{-}DPGYIGSR\text{-}NH_2$	-	90/62	85/50	-	-	-	-

[a] Type of linkage between oligonucleotide and conjugated moiety: **T** = thiazolidine, **O** = oxime, **H** = hydrazine.

Scheme 2[a]



[a] R^1, R^2 = unprotected oligonucleotide chain; R = conjugated moiety (Table 1).

The hydrolytic stabilities of thiazolidine and oxime conjugates were studied as a function of pH. Purified conjugates were incubated in a suitable buffer for 24 h and then analyzed by RP-HPLC in ion-pair mode (Table 2). Conjugates proved to be stable over a wide pH range, thiazolidine being more stable than oxime.

Initially, we chose to use 4-hydrazinobenzoic acid as a model compound and N-terminal hydrazine modification of peptides (*24*, *29*). However, use of 4-Fmoc-hydrazinobenzoic acid, which is commercially available, may give rise to side-products involving acylation of unprotected nitrogen atoms during solid-phase peptide synthesis under forcing conditions. Therefore, we decided to use our recently developed *S*-benzyl thiosuccinate reagent originally proposed for Fmoc-based solid-supported peptide N-terminal thioester synthesis (*30*). *S*-Benzyl thiosuccinic acid was coupled at the end of normal Fmoc-on solid-phase synthesis by the same HATU-mediated coupling cycle as used for a standard Fmoc amino acid. Then, the resin was treated manually with 0.5 M hydrazine hydrate solution in dioxane for 2 h at room temperature, and, after washing and drying, subjected to normal TFA cleavage. The resulting peptide N-terminal succinyl monohydrazide **9** was analyzed by RP-HPLC and found to be >95% pure and had the correct mass as judged by MALDI-TOF mass spectrometry.

Peptides were conjugated to oligonucleotides under the conditions reported above that were optimized for conjugation of small molecules. The main problem during conjugate synthesis was found to be product isolation and purification, especially in the case of cationic peptides **6** and **7**. Various methods were tried, such as NAP-5 desalting, precipitation by acetone, ethanol, or *n*-butanol, and dialysis, but all of these methods led often to considerable losses of final product. Also we were unable to purify conjugates of peptides **6** and **7** by direct RP-HPLC. In general, the best results were obtained by use of denaturing PAGE, and therefore this method of initial purification was adopted routinely for all conjugates. Analyses of PAGE-purified conjugates by RP-HPLC (ion-pair mode) and by MALDI-TOF MS are presented in Table 3, and a typical HPLC trace is shown in Figure 2.

Table 2. % Hydrolysis of Thiazolidine and Oxime Conjugates at Various pH

	pH					
conjugate[a]	2.0	3.0	4.0	8.0	9.0	10.0
I.1	100	5	<5	<5	10	55
I.2	100	10	<5	<5	30	>90

[a] Oligonucleotides are designated by roman numbers, conjugated molecules by arabic (Table 1).

In the case of the doubly modified 2′-deoxyoligonucleotide III, we were unable to isolate conjugates with cationic peptides (conjugates **III.6** and **III.7)** since during the reaction a white precipitate was formed. This precipitate was insoluble in 4 M NaCl or 2 M $LiClO_4$ and only marginally soluble in DMSO. Further studies will be required to identify more suitable solvent conditions for conjugation of these more demanding peptides. However, conjugates of the singly modified 2′-deoxyoligonucleotide **I** with cationic peptides **6** and **7** were isolated in reasonable yield. Further, good yields were obtained for both singly and doubly modified oligonucleotides **II** and **III** and for singly and doubly modified 2′-*O*-methyloligoribonucleotides **IV**, **V**, and **VI** in conjugation with a model laminin peptides through either oxime or hydrazine linkages (peptides **8** and **9**).

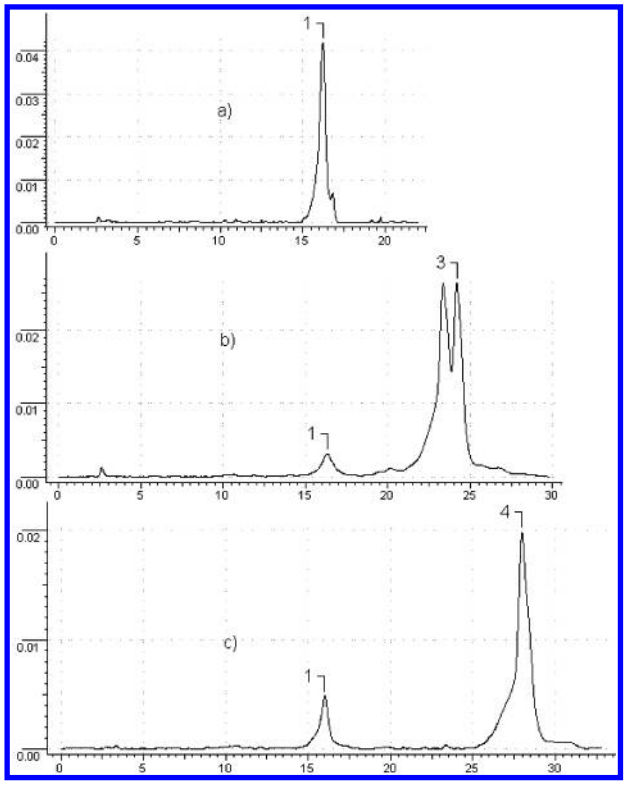


Figure 1. (a) Starting oligonucleotide **VII** (1); (b) reaction mixture of **VII** with *O*-benzylhydroxylamine **2**, peaks 2 and 3 are likely *E*,*Z*-isomers of the conjugate **VII.2**; (c) $NaBH_3CN$ reduction of **VII.2**, peak 4 is the reduced conjugate.

Table 3. RP-HPLC (ion-pair mode) and MALDI-TOF MS Analysis of Oligonucleotides and Conjugates

compound[a]	HPLC retention time,[b] min	calcd mass	found mass
I	13.1	3642.4	3644.6
I.6	14.3	4970.0	4972.7
I.7	14.0	5346.4	5349.3
II	13.1	3642.4	-
II.8	15.5	4528.3	-
II.9	14.9	4571.4	4570.6
III	12.9	3718.4	3717.4
III.8	17.5	5490.3	5486.8
III.9	16.0	5576.4	5575.3
IV	15.7	3972.7	3965.0
IV.4	15.9	4100.8	-
IV.8	18.9	4845.6	4844.8
V	15.6	3972.7	3967.6
V.4	16.0	4100.8	-
V.8	19.7	4845.6	4843.3
VI	15.0	4018.7	4014.3
VI.4	15.7	4275.0	-
VI.8	22.1	5806.5	5802.6

[a] Oligonucleotides are designated by roman numbers, conjugated molecules by arabic (Table 1). [b] RP-HPLC in ion-pair mode (for details, see Experimental Section).

A number of conjugates with various hydrazides, hydrazines, and carbazates were synthesized (Table 4), including reporter group (biotin), marker groups (acridine, pyrene), terephthalic dihydrazide that can be used for oligonucleotide cross-linking, and several lipophilic groups.

We have also studied the influence of 2′-modification and 2′-peptide conjugation on the stability of duplexes formed with complementary DNA and RNA targets. It has been shown previously that incorporation of a similarly modified 2′-*O*-(2,3-dihydroxypropyl)thymidine (*58*,*59*) decreased duplex stability with complementary

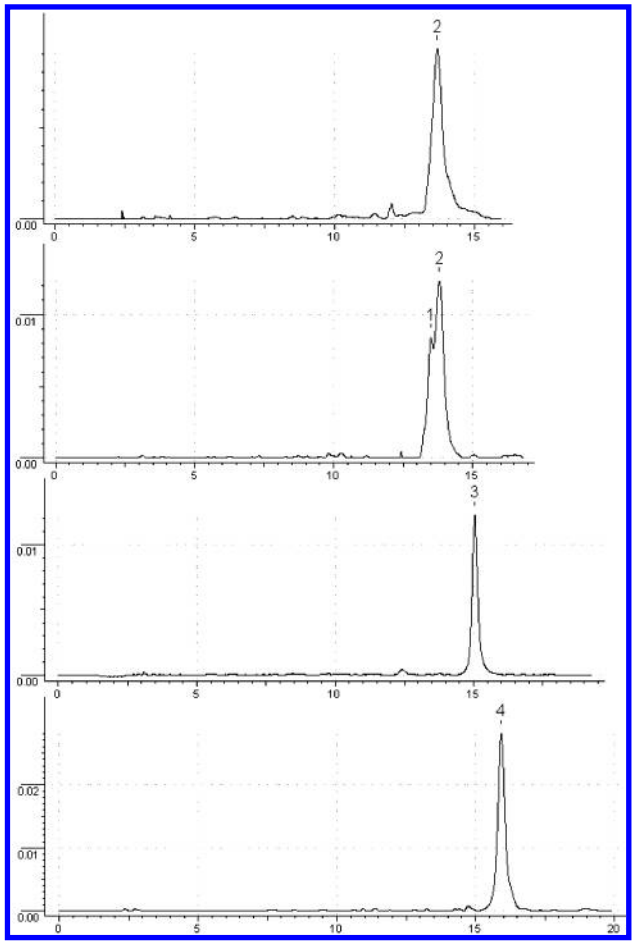


Figure 2. (a) 2′-Aldehyde **III** (2); (b) coinjection of 2′-aldehyde **III** (2) with parent 1,2-diol (1); (c) purified conjugate **II.9** (3); (d) purified conjugate **III.9** (4).

DNA (ΔT_m/mod = −2.2 °C) but increased stability with complementary RNA (ΔT_m/mod = +0.8−1.5 °C). It should be noted that here we have used the corresponding modified U derivative and that replacement of thymidine by uridine of itself would be expected to lead to a duplex T_m decrease of about 0.5 °C (*59*). With this in mind, the thermal melting results of duplexes formed with complementary DNA and RNA targets are summarized in Table 5. In general, the results are in accordance with published data (*5*, *8*). Thus in the case of a DNA target, diol (lines 2−4) or aldehyde modification (lines 5−7) resulted in moderate destabilization of about 3 to 4 °C per residue modified. Very similar destabilizations were found for conjugates with a dipeptide Cys-Gly (lines 8−10), aminooxyacetic acid (lines 11−13) and with a model laminin peptide conjugated through either oxime (lines 14 and 15) or hydrazine linkages (lines 16 and 17). Conjugation of two peptides (**III.8**, **III.9**) decreased duplex stability by about twice the amount seen for conjugation with a single peptide (**II.8**, **II.9**). By contrast in the case of the RNA target, diol modifications were slightly stabilizing (lines 2−4), whereas aldehyde modifications were broadly neutral (lines 5−7, taking into consideration the U for T substitution). Slight stabilization was seen for dipeptide (lines 8−10) and acetic acid conjugates (lines 11−13). Single laminin peptide conjugation (lines 14 and 16) resulted in duplexes melting some 2.8 to 3.1 °C higher, but slightly less stabilization per modification was seen for the double laminin peptide conjugates (lines 15 and 17). The nature of the linkage between peptide and oligonucleotide fragments had little or no influence on

Table 4. Examples of Oligonucleotide II Hydrazine Conjugates with Small Molecules

conjugate[a]	conjugated molecule	MALDI-TOF MS, calcd/found	HPLC retention time,[b] min	yield, %
II.10	semicarbazide	3708.5/3709.4	14.05	79
II.11	methyl carbazate	3723.5/3725.1	14.71	85
II.12	4-hydroxyphenyl hydrazide	3785.6/3787.3	15.08	93
II.13	biotin hydrazide	3853.7/3853.4	14.01	87
II.14	2-aminophenyl hydrazide	3784.6/3784.6	18.14	92
II.15	benzyl hydrazide	3731.5/3729.4	16.41	89
II.16	4-methoxyphenyl hydrazide	3799.6/3799.7	17.74	75
II.17	*n*-pentanoic hydrazide	3763.6/3763.6	21.36	95
II.18	9-hydrazinoacridine	3804.6/3802.3	18.69	80
II.19	1-pyrenebutyric hydrazide	3935.8/3939.2	-[c]	84
II.20	benzyl carbazate	3761.5/3761.9	19.37	70
II.21	3-hydroxy-2-naphthydrazide	3835.7/3839.8	21.74	72
II.22	terephthalic dihydrazide	3789.6/3787.5	14.74	81
II.23	terephthalic dihydrazide acetone adduct	3829.6/3831.2	16.14	74

[a] Oligonucleotides are designated by roman numbers, conjugated molecules by arabic (Table 1). [b] RP-HPLC in ion-pair mode (for details see Experimental Section). [c] Compound **II.19** was purified by RP-HPLC due to high lipophilicity.

Table 5. Thermal Stabilities of Duplexes Formed by 2′-Modified Oligonucleotides and Conjugates with Complementary DNA and RNA Targets

		DNA d(TGAGCCTGGAG)		RNA r(UGAGCCUGGGAG)	
	oligonucleotide or conjugate	$T_m \pm 0.2$, °C	ΔT_m/mod,[a] °C	$T_m \pm 0.2$, °C	ΔT_m/mod[a], °C
1	d(CTCCCAGGCTCA)	56.0	-	58.6	-
2	**I**[b]	52.3	−3.7	59.0	+0.4
3	**II**[b]	52.9	−3.1	59.2	+0.6
4	**III**[b]	49.5	−3.3	59.7	+0.6
5	**I**	51.0	−5.0	58.2	−0.4
6	**II**	51.7	−4.3	58.3	−0.3
7	**III**	46.9	−4.6	57.7	−0.5
8	**I.4**	52.9	−3.1	58.5	−0.1
9	**II.4**	53.1	−2.9	58.7	+0.1
10	**III.4**	50.1	−3.0	58.7	−0.1
11	**I.5**	52.5	−3.5	59.0	+0.4
12	**II.5**	52.1	−3.9	59.1	+0.5
13	**III.5**	48.6	−3.7	59.9	+0.7
14	**II.8**	54.1	−1.9	61.7	+3.1
15	**III.8**	50.6	−2.7	60.6	+1.0
16	**II.9**	53.9	−2.1	61.4	+2.8
17	**III.9**	50.6	−2.7	60.5	+1.0

[a] $\Delta T_m/\text{mod} = [T_m(n) - T_m(1)]/m$, where m is the number of modifications in a duplex. [b] Modified oligonucleotide containing 2′-*O*-(2,3-dihydroxypropyl)uridine.

duplex stability (compare lines 14 with 16 and 15 with 17).

Finally, we have studied the influence of 2′-peptide conjugation on binding of a 12-mer 2′-*O*-methyloligoribonucleotide to its complementary sequence in the apical stem-loop of the HIV-1 TAR RNA (*53, 54*). Under the buffer conditions of transcription (see Experimental Section), single laminin conjugates **IV.8** and **V.8** were insignificantly affected in K_d of binding to the structured TAR RNA target (3.1 ± 0.2 and 4.3 ± 0.9 nM, respectively) compared to the unmodified 2′-*O*-methyloligoribonucleotide (3.2 ± 0.7 nM) (*54*). The double laminin conjugate was only about 2-fold increased in K_d (6.5 ± 0.7 nM). Single and double laminin conjugates of 12-mer 2′-*O*-methyloligoribonucleotides (**IV.8**, **V.8**, and **VI.8**) were tested also for their ability to inhibit Tat-dependent in vitro transcription on an HIV-1 DNA template in the presence of HeLa cell nuclear extract (*54*). The results showed (Figure 3) that the two single laminin conjugates were almost identical in their ability to inhibit Tat-dependent transcription compared to the unconjugated 2′-*O*-methyloligoribonucleotide. For the double laminin conjugate, the inhibition was a little less pronounced at the lower concentrations, but at higher concentrations above 600 nM there was an increased inhibitory power. Control in vitro transcription using a CMV promoter showed that this double laminin conjugate also had significant nonspecific inhibition of constitutive transcriptional activity, particularly above 600 nM, but single laminin conjugates showed only a small nonspecific inhibitory activity (data not shown). It should be borne in mind that the laminin peptide sequence was chosen only as a model and not for any other property. Thus, the nonspecific transcriptional inhibition activity is likely to be inadvertent.

In conclusion, we have shown that oligonucleotides containing 2′-aldehydes generated from 2′-*O*-(2,3-dihydroxypropyl)uridine residues may be conjugated singly or multiply with unprotected peptides by nucleophilic addition−elimination reactions to form thiazolidine, oxime, or hydrazone linkages. Reactions are carried out at pH 4.0−4.5 for thiazolidine and 4.5−5.0 for oxime and hydrazone. The peptide excess can be reduced to 1.2 to 1.8 equiv, and reaction is best carried out at 37 °C in the presence of an organic cosolvent. Similar oxime and thiazolidine conjugation chemistry has been reported recently for the attachment of peptides to 5′-aldehyde-substituted oligonucleotides (*22*). The advantage of our 2′-aldehyde conjugation route is the ability in principle to conjugate more than one peptide at specific locations along the oligonucleotide chain. Further, the 5′- and 3′-termini of the oligonucleotide remain free for attachment of other labels, such as fluorescent or radioactive, or enzyme-catalyzed transformations. In addition, we have

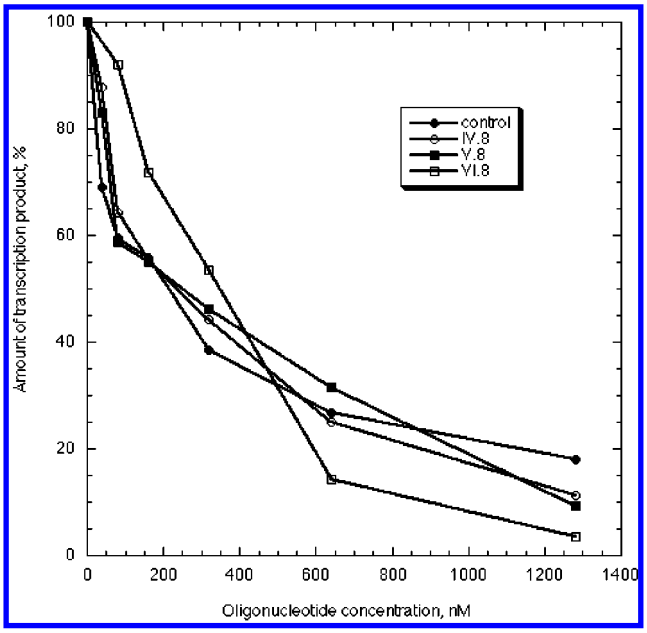


Figure 3. 2′-*O*-Methyloligonucleotide–peptide conjugate inhibition od Tat-dependent in vitro transcription in HeLa cell nuclear extract. Amount of full-length transcript is depicted as a function of oligomer concentration (see inset for key). Control is 2′-*O*-methyldodecamer CUCCCAGGCUCA.

explored a third conjugation procedure by formation of hydrazone linkages, which may be conveniently reduced to hydrazine linkages by use of a solid-supported borohydride reagent.

We have found that for the peptide and small molecule models studied, the resultant peptide–oligonucleotide conjugates generally bind to complementary RNA targets with equally high or better affinity than the corresponding unmodified, unconjugated oligonucleotides. Further, single and double model laminin peptide conjugates of 2′-*O*-methyloligoribonucleotides retained their ability to bind to a structured TAR RNA target and to inhibit HIV-1 Tat-dependent in vitro transcription. Therefore, this 2′-aldehyde conjugation route should be suitable for general use in the design of conjugates of oligonucleotide analogues antisense to RNA targets. We are now exploring these conjugation routes for the attachment of cell-penetrating and cell-targeting peptides in single or multiple copies for cell delivery studies.

ACKNOWLEDGMENT

The authors thank A. V. Kachalova, E. M. Volkov, and Dr. E. M. Zubin for their suggestions and helpful discussions. This work was supported by Wellcome Trust grant 057361, and partially by RFBR grant 00-04-48260.

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