Efficient Conjugation of Peptides to Oligonucleotides by "Native Ligation"

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Received February 15, 2000

A new strategy has been developed for conjugation of peptides to oligonucleotides. The method is based on the "native ligation" of an N-terminal thioester-functionalized peptide to a 5′-cysteinyl oligonucleotide. Two new reagents were synthesized for use in solid-phase peptide and oligonucleotide synthesis, respectively. Pentafluorophenyl *S*-benzylthiosuccinate was used in the final coupling step in standard Fmoc-based solid-phase peptide assembly. Deprotection with trifluoracetic acid generated in solution peptides substituted with an N-terminal *S*-benzylthiosuccinyl moiety. *O-trans*-4-(*N*-α-Fmoc-*S*-*tert*-butylsulfenyl-L-cysteinyl)aminocyclohexyl *O*-2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite was used in the final coupling step in standard phosphoramidite solid-phase oligonucleotide assembly. Deprotection with aqueous ammonia solution generated in solution 5′-*S*-*tert*-butylsulfenyl-L-cysteinyl functionalized oligonucleotides. Functionalized peptides and oligonucleotides were used without purification in native ligation conjugation reactions in aqueous/organic solution using tris-(2-carboxyethyl)phosphine to remove the *tert*-butylsulfenyl group in situ and thiophenol as a conjugation enhancer. A range of peptide−oligonucleotide conjugates were prepared by this route and purified by reversed-phase HPLC.

Introduction

Oligonucleotides and their analogues are used widely as sequence specific reagents to block gene expression of RNA within cells.[1,2] However, oligonucleotide efficacy in cell culture is often limited by poor cellular uptake.[3,4] Among the many molecules that have been reported to enhance cell delivery of oligonucleotides are a number of peptide carriers. Some of these peptides have been used as additives to aid transfection.[5−11] There are also several reports of oligonucleotides conjugated covalently to peptides for delivery to cell lines in culture.[12−15] However, a recent study showed that some peptide−oligonucleotide conjugates are inactive as antisense agents because of entrapment within endosomes.[16] Systematic studies of the sequence and structural requirements for good cell penetration and compartmentalization in a range of cell lines as well as correlation with biological activity have not yet been reported for peptide−oligonucleotide conjugates. This is because such studies have been hampered by the often cumbersome and inefficient methods required for the chemical synthesis of such bioconjugates.

Total stepwise solid-phase approaches on a single support have posed serious difficulties because of incompatibilities in peptide and oligonucleotide protection and assembly chemistries. Although some promising routes have been suggested, so far only a restricted range of peptide−oligonucleotide conjugates have been prepared and the methods have been mostly unsuitable for the synthesis of conjugates containing peptides of length and type suitable for cell delivery studies.[17−25] A post-as-

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10.1021/jo000214z CCC: $19.00
Published on Web 07/15/2000

sembly conjugation strategy has found much more general applicability. Here, peptide and oligonucleotide moieties are assembled separately on their own solid supports. Each biomolecule is designed to carry a reactive functionality that is released upon full deprotection and cleavage from the support. Peptide and oligonucleotide parts are then joined in aqueous solution through these reactive functionalities by a selective reaction. To date, only a limited number of such reactions has been described, for example the formation of a disulfide bond,[12,26,27] reaction of a cysteine peptide with a maleimido oligonucleotide,[28−30] or a bromoacetyl peptide with a thiol-functionalized oligonucleotide.[31]

These established routes suffer from certain disadvantages. For example, a disulfide bond is unstable to reducing agents that may be present under many assay conditions or within cells. The maleimide−thiol route of conjugation requires a functionalization step on the oligonucleotide part after release from the solid support. Two of the routes present limitations in peptide sequence (requirement for a cysteine). Finally, post-assembly joining routes may sometimes lead to inefficient conjugation, due to secondary structure or poor solubility of certain peptide components in aqueous solution, and this may be relieved in some cases by the addition of denaturants. However, a major advantage of post-assembly conjugation is that all peptide side chains and nucleobases are deprotected before conjugation, and thus there is no problem of compatibility of assembly and deprotection chemistries. An interesting variation of the solution phase strategy is where both peptide and oligonucleotide fragments are released from their respective supports still carrying side-chain and nucleobase protection, purified and conjugated under nonaqueous peptide-bond-forming conditions.[32,33] Although encouraging, this route is not yet validated for a wide-range of peptides carrying side-chain protecting groups that require deprotection following conjugation.

We have reported recently a solid-phase peptide fragment coupling strategy, which was precedented by a previous report of a tripeptide conjugation by Grandas et al.[34] In our studies, oligonucleotide and peptide moieties were assembled separately on their own supports but only the peptide fragment was removed from its support and purified as a peptide that now carries only N_α-Fmoc protection. We then explored the solid-phase conjugation of such peptide fragments under various peptide bond-forming conditions. Here, the C-terminus of the peptide fragment was reacted with support-bound oligonucleotides that had been 5′-functionalized on-column with a primary amino group linker.[35,36] After cleavage from the support, the conjugate was deprotected and analyzed. By this route, we were able to obtain efficient conjugation for small peptides, especially if 2-cyanoethyl phosphate protecting groups were removed from the oligonucleotide prior to conjugation and if the spacer distance between oligonucleotide and amino group was 6 carbon atoms or more. However, longer hydrophobic or highly basic peptide fragments were poorly conjugated under these conditions.[36]

We decided to search for alternative conjugation chemistries that might proceed both with high yield and selectivity for preparation of peptide conjugates of length and variety useful in cellular uptake studies. Such chemistries should be adaptable to a range of solvent conditions in order to maintain solubility of peptides and oligonucleotides that vary substantially in hydrophobicity and charge. Such solubility may depend not only on the intrinsic sequence but also on the level of chemical protection that remains on each biomolecule and the nature of any analogue incorporated. We also wished any functionalization method to be compatible with mild Fmoc-based peptide assembly chemistry and a wide range of side-chain protecting groups. Further, we wished the functionalities to be introduced on to each biomolecule by techniques that could be carried out simply and on-column, without the need for extended manipulation following release from the solid supports.

In the search for suitable conjugation chemistry, a method of protein synthesis came to our attention that involves "native ligation" of two largely unprotected peptide fragments, one containing a C-terminal thioester and the other an N-terminal cysteine.[37] This technique has been applied recently to numerous interesting protein syntheses.[38,39] This conjugation method is carried out in aqueous solution but has the advantage that efficient coupling is also achieved in the presence of denaturing agents and organic solvent additives and thus is suitable in principle for both hydrophobic and hydrophilic peptides. As we began our studies, the only reported peptide−oligonucleotide conjugation procedure based on a ligation approach was the coupling of a 3′-amino oligonucleotide to a peptide thioester oligonucleotide aligned on a DNA template.[40] However, the requirement for three separate oligonucleotide chains, one of which must carry a 3′-amino group, makes this synthesis route highly impractical and is further restricted to 3′-peptide conjugated oligonucleotides. Very recently, the native ligation of a 5′-thioester functionalized RNA with a Cys-Gly dipeptide and with a 13-mer cysteine-containing peptide was reported.[41]

We have now developed a novel and general method of peptide−oligonucleotide conjugation based on the principle of "native ligation" which is template free. The method involves conjugation of a peptide fragment as an

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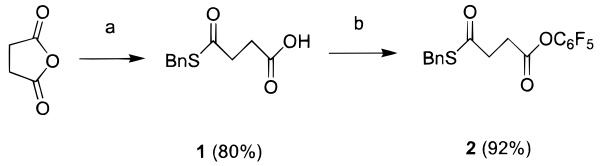
Scheme 1[a]



[a] Key: (a) BnSH, DMAP, pyridine; (b) DCC, pentafluorophenol, CH_2Cl_2.

N-terminal thioester to an oligonucleotide functionalized at the 5′-end with a cysteine moiety.

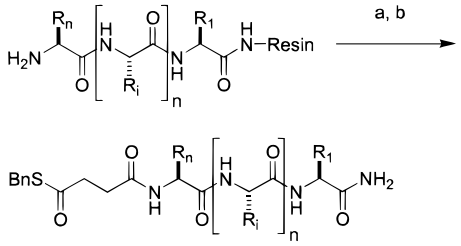
Results

For protein synthesis by native ligation, it is common for C-terminal thioesters of peptide fragments to be prepared. Such C-terminal thioesters have been obtained primarily by Boc/benzyl peptide assembly chemistry on specially designed solid supports either through alkylation of a thio acid intermediate[42] or more recently by a direct route.[43] Use of the milder Fmoc/*tert*-butyl chemistry for peptide assembly has been hampered by the instability of polymer-anchored thioesters to repetitive piperidine treatment required for N-terminal Fmoc removal. The use of less nucleophilic secondary amines for N-deprotection as well as an HOBt amine scavenger has improved yields somewhat.[44] But, in general, post-assembly introduction of a thioester is necessary.

No suitable route for introduction of a C-terminal thioester following peptide assembly by the Fmoc/*tert*-butyl method has been reported until very recently.[45] Therefore to enable initial studies of peptide−oligonucleotide conjugation via native ligation, it was simpler to develop a method for synthesis of N-terminal thioesters of peptide fragments. For this purpose we designed a new reagent, pentafluorophenyl *S*-benzylthiosuccinate **2**, which is prepared in two steps. First, reaction of succinic anhydride with benzyl mercaptan produced *S*-benzylthiosuccinic acid in 80% yield, which was converted into the corresponding pentafluorophenyl ester in 92% yield (Scheme 1).

The thiosuccinate **2** may be incorporated into a peptide fragment following standard Fmoc/*tert*-butyl peptide assembly on a commercially available peptide synthesizer (Scheme 2). In order that the C-terminus of the peptide is protected from possible interference with native ligation, we have utilized a PEG-polystyrene support containing a standard Rink amide linker or a PAL linker. Thus N-terminal benzyl thioester peptide is released into solution from the solid support as a C-terminal amide during side-chain deprotection by treatment with TFA/phenol/benzyl mercaptan/water (92.5:2.5:2.5:2.5 v/v). A range of peptides containing N-terminal *S*-benzylthiosuccinate was assembled and deprotected. Each peptide product was assayed for purity by reversed-phase HPLC as well as for the correct mass of the major product by MALDI-TOF mass spectrometry (Table 1). It is important

Scheme 2[a]



[a] Key: (a) either **2** (4.5 equiv), HOAt (1 equiv), DMF, 4 h, rt, or **1** (5 equiv), HATU (4.5 equiv), DIEA (10 equiv), DMF, 1 h, rt; (b) TFA−BnSH−PhOH−H_2O (90:5:2.5:2.5 v/v/v/v), 1−6 h, rt.

to note that no evidence was found for loss of the *S*-benzyl group from the thiosuccinate during the acidic deprotection step.

One advantage of preparation of N-terminal thioesters is that the thioester group is spaced away from the terminal amino acid. This is helpful in that C-terminal thioesters of peptides containing sterically hindered amino acids at the C-terminus, such as threonine, isoleucine, valine or proline, are known to couple only very slowly in native ligation reactions.[43] A second advantage is that there is no possibility of peptide racemization during conjugation reactions.

To obtain oligonucleotides containing a cysteine moiety, we synthesized a novel phosphoramidite reagent **5** in two simple steps (Scheme 3). Thus, *trans*-4-aminocyclohexanol was acylated by reaction with the pentafluorophenyl ester of *N*-α-Fmoc-S-*tert*-butylsulfenylcysteine in 82% yield. The corresponding phosphoramidite was prepared by subsequent standard phosphitylation chemistry and purified by column chromatography in 66% yield. Even higher yields (95%) were obtained in preparation of the corresponding *S*-trityl-protected cysteine phosphoramidite **6**.

Whereas many commercially available phosphoramidite reagents used for 5′- functionalization of oligonucleotides that utilize short linear alkyl chains are oils, phosphoramidite **5** containing the *trans*-4-aminocyclohexanol moiety, a secondary alcohol, is a solid and is much more stable. Phosphoramidite **5** was used in the final step of coupling following standard oligonucleotide assembly on a controlled pore glass solid-support by the phosphoramidite method (1 μM nucleoside bound to the support) using a commercial DNA/RNA synthesizer. A number of oligonucleotides were prepared in this way, deprotected at the nucleobases and released into solution by treatment with concentrated aqueous ammonia solution at 55 °C. The *S*-*tert*-butylsulfenyl protecting group is maintained but the N_α-Fmoc group is removed from the cysteine moiety during ammonia treatment. The cysteine-modified oligonucleotides were characterized by ion exchange and/or reversed phase HPLC and by MALDI-TOF mass spectrometry (Table 2). Average coupling yields of phosphoramidites **5** and **6** were >97% as judged by HPLC analysis following deprotection.

As a first trial, we then evaluated the conjugation in aqueous solution of a peptide containing an N-terminal thioester to an oligonucleotide containing a 5′-cysteine (Scheme 4). We chose in each case a peptide and an oligonucleotide fragment, containing their respective functionalities, that had been assembled in high yield as judged by HPLC analysis, and that could be used therefore without the need for HPLC purification. We

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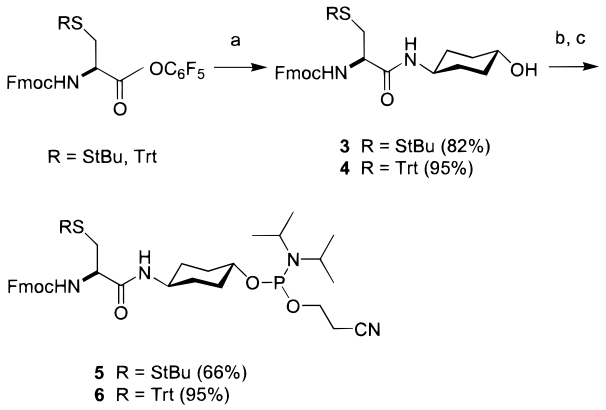
Table 1. Synthesis of Peptide N-Terminal Thioesters[a]

peptide sequence[b]	MALDI-TOF MS[c]	HPLC retention time, min	purity of crude product,[d] %	yield,[e] %
PTSQSRGDPTGPKE P1	1660.2 (1660.8)	13.6	98.5	86.6[f]
Sar-Leu-Gly-Ile-Gly **P2**	657.9 (657.8)	17.6	92.2	89.9[f]
ALPPLERLTL **P3**	1325.7 (1326.7)	19.6	74.7	43.3
GALFLGFLGAAGSTMGAWSQPKSKRKV **P4**	2969.1 (2969.6)	21.6	48.3	25.0
PQIK(Tfa)IWFPNRRK(Tfa)PFK(Tfa)K(Tfa) **P5**	2715.0 (2715.9)	22.4	72.0	44.2
GRK(Tfa)K(Tfa)RRQRRR	1791.6 (1792.9)	15.1	76.5	38.1
DRVIEVVQGAYRAIRNIPRRIRQG	3040.6 (3039.7)	20.0	28.6	17.8
QAKKKKLDK	1289.9 (1290.8)	11.9	90.3	68.9

[a] Abbreviation used: Tfa = trifluoroacetyl. [b] All the peptides contain an N-terminal *S*-benzyl thiosuccinyl group and are C-terminal amides. [c] Values in parentheses are calculated. [d] Integrated from HPLC traces. [e] Calculated from resin mass and loading indicated by supplier. [f] Not purified.

Scheme 3[a]



[a] Key: (a) *trans*-4-aminocyclohexanol hydrochloride, Et_3N, DMF; (b) 2-cyanoethyl-*N*,*N*-diisopropylamino chlorophosphine, DIEA, CH_2Cl_2 (**5**); (c) 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropyl phosphordiamidite, diisopropylammonium tetrazolide, CH_2Cl_2 (**6**).

found that it was possible to avoid the necessity for prior removal of the *S*-*tert*-butylsulfenyl protecting group from the peptide fragment by in situ reduction with the water soluble reducing agent tris(2-carboxyethyl)phosphine (TCEP)[46] during the native ligation reaction. To facilitate thiol exchange and to improve reactivity, excess thiophenol was also added to the conjugation reaction.[47] The conjugation was carried out for 24 h at room temperature between a 14-mer peptide P1 (2.5 μmol) and a 5-mer oligonucleotide of thymidine ODN1 (0.5 μmol) in 1 mL of 0.1 M phosphate buffer (pH 7.5) in the presence of 7 M urea (conditions A, Table 3and Figure 2). The products were separated by reversed-phase HPLC, which showed predominantly a single product of conjugate with a slightly later elution time compared to the starting oligonucleotide (Figure 1a). Following HPLC purification, the product (75% isolated yield starting from unpurified oligonucleotide) was analyzed by MALDI-TOF mass spectrometry and found to have the expected mass for the desired peptide−oligonucleotide conjugate.

As a second example, the reaction products of conjugation of excess 14-mer peptide N-terminal benzyl thioester **P1** with a 5′-cysteinyl 15-mer mixed sequence phosphodiester oligodeoxyribonucleotide ODN2 were separated by reversed phase HPLC (Figure 1b). The conditions of conjugation were identical except that the 7 M urea was omitted (conditions B). This showed that all the cysteinyl oligonucleotide had reacted to give a major product with later elution time. Following HPLC purification, the product was analyzed by MALDI-TOF mass spectrometry and found to have the expected mass for the desired peptide−oligonucleotide conjugate. The isolated yield of conjugate based on the amount of unpurified oligonucleotide was 65% (Table 3).

These first two successful examples of peptide−oligonucleotide native ligation involved use of a peptide **P1** that contained an N-terminal proline. One possible concern of the use of the *S*-benzylthiosuccinate linker was that under basic conditions a cyclization side-reaction could take place, involving attack of a deprotonated N-terminal amido group of the peptide on the thioester carbonyl group. Such a side reaction cannot take place in the case of N-terminal proline, since the amido group is secondary and does not contain an acidic hydrogen atom. To determine whether such a side reaction can take place we chose a particularly challenging conjugation of a long 27-mer peptide **P4** that contains an N-terminal glycine, an unhindered amino acid that would be the most likely to be prone to cyclization. Peptide **P4** contains both a long hydrophobic section as well as a C-terminal basic region. Thus, peptide **P4** (2.5 μmol) and oligonucleotide **ODN2** (0.5 μmol) were incubated at pH 7.5 in the absence of denaturing agent, but in the presence of **TCEP** and thiophenol. After 48 h at room temperature, HPLC examination showed that no conjugation product was obtained. Instead, MALDI-TOF mass spectra of the crude reaction mixture showed that most of the peptide **P4** had been converted into two products, one consistent in mass with N-terminal cyclization and loss of the thioester moiety and the other consistent with the free carboxylic acid hydrolysis product (data not shown).

To circumvent the cyclization reaction and also to investigate alternative parameters for conjugation, we chose two further peptide conjugation examples. A peptide containing an N-terminal sarcosine (*N*-methyl glycine) that cannot undergo cyclization, peptide **P2**, was conjugated to oligonucleotide **ODN2** and also to **ODN3**. In the conjugation to **ODN2**, acetonitrile was used as cosolvent (conditions **C**), whereas DMF was cosolvent in conjugation to ODN 3 (conditions **D**). The concentrations of peptide and oligonucleotide were 10-fold lower in each case. In addition, since we noticed that the rate of reduction by TCEP may be pH-dependent, we carried out the **ODN2** conjugation reaction at pH 6.5 (conditions C) for 48 h and in the case of **ODN3** a prereduction for 3 h by TCEP at pH 6.5 was followed by alteration of the pH to 7.5 and addition of the peptide component for a reaction time of 48 h (conditions **D**). In these cases the major products of the reactions were the expected con-

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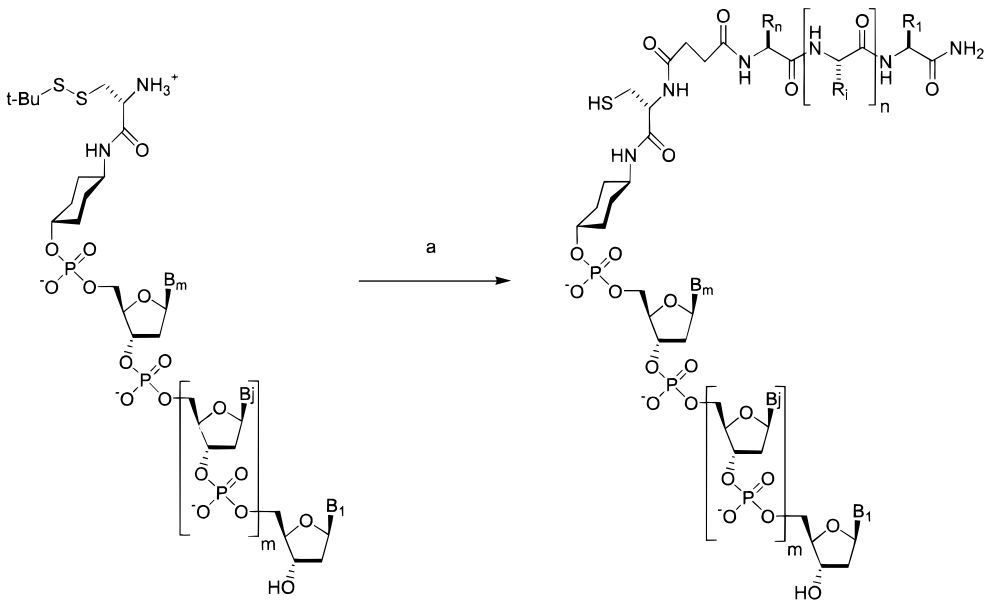
Table 2. Synthesis of 5′-Cysteinyloligonucleotides[a]

oligonucleotide sequence[b]	MALDI-TOF MS[c]	HPLC retention time, min	purity of crude product,[d] %	isolated yield,[e] %
H-Cys(StBu)-TTT TT	1827.1 (1827.4)	16.6	87.1	76.3
H-Cys(Trt)-TTT TT	1983.2 (1982.6)	22.1	76.6	72.5
H-Cys-TTT TT	1738.2 (1739.4)	14.8	82.2	ND
H-Cys(StBu)-CTC CCA GGC TCA AAT	4868.0 (4866.4)	15.0	82.6	61.6
H-Cys(StBu)-GCT CCC AGG CTC AAA	4888.1 (4888.9)	14.4	89.7	66.8
H-Cys(StBu)-AGC TCC CAG GCT CAA	4888.0 (4888.9)	17.0	86.5	60.3
H-Cys-GCT CCC AGG CTC AAA	4803.1 (4804.0)	13.7	100	ND
H-Cys(Trt)-CTC CCA GGC TCA AAT	5017.1 (5018.0)	21.5	91.2	72.2
H-Cys(Trt)-GCT CCC AGG CTC AAA	5043.7 (5043.0)	20.8	89.3	68.4
H-Cys(Trt)-AGC TCC CAG GCT CAA	5043.9 (5043.0)	20.7	90.5	70.1

[a] Abbreviations used: StBu = *tert*-butylsulfenyl, Trt = triphenylmethyl, ND = not determined. [b] All the oligonucleotides contain 5′-terminal *S*-protected or unprotected trans-*N*-(L-cysteinyl)aminocyclohexyl phosphate group (denoted as Cys). [c] Values in parentheses are calculated. [d] Integrated from HPLC traces. [e] A_{260} units obtained from 1 μmol synthesis.

Scheme 4[a]



[a] Key: (a) peptide thioester (5 equiv), 100 mM TCEP, excess PhSH under various solvent and buffer conditions (see the Experimental Section), rt, 24 h.

Table 3. Synthesis of Peptide-*N*-to-5′-oligonucleotide Conjugates

oligonucleotide sequence[a]	peptide sequence[a]	method	MALDI-TOF MS[b]	HPLC retention time, min	yield,[c] %
TTT TT **ODN1**	PTSQSRGDPTGPKE **P1**	**A**	3276.7 (3275.6)	15.8	75
GCT CCC AGG CTC AAA **ODN2**	PTSQSRGDPTGPKE **P1**	**B**	6340.2 (6340.5)	15.6	65
GCT CCC AGG CTC AAA **ODN2**	Sar-Leu-Gly-Ile-Gly **P2**	**C**	5349.0 (5349.1)	17.6	26[d]
GCT CCC AGG CTC AAA **ODN2**	ALPPLERLTL **P3**	**C**	6043.4 (6044.5)	20.1	22
AGC TCC CAG GCT CAA **ODN3**	Sar-Leu-Gly-Ile-Gly **P2**	**D**	5348.5 (5349.1)	17.4	43[e]
AGC TCC CAG GCT CAA **ODN3**	ALPPLERLTL **P3**	**D**	6043.9 (6044.5)	19.8	51

[a] All the peptide−oligonucleotide conjugates are linked from the N-terminus to the 5′-end via (*N*-succinyl-L-cysteinyl)-*trans*-aminocyclohexyl phosphate group. [b] Values in parentheses are calculated. [c] Calculated from A_{260} units of starting crude oligonucleotide. [d] Additionally, 17% of mixed disulfide with thiophenol was isolated. [e] 14% of mixed disulfide with thiophenol was isolated.

jugates as seen from HPLC, which were isolated in 26 and 43% yields respectively (Table 3). There was also in each case a side product of the mixed disulfide of conjugate with thiophenol. A hydrophobic 10-mer peptide **P3**, which has a N-terminal alanine and which has the sequence corresponding to a nuclear export signal was also conjugated to **ODN2** and **ODN3** under the same conditions as above. Once again the major product by HPLC was the desired conjugates which were isolated in 22 and 51% yields, respectively. No cyclized product was detected in conjugations with peptide **P3**. In each case the product conjugate showed a single peak after HPLC purification and the correct MALDI-TOF mass (Table 3).

Discussion

Efficient and convenient procedures for conjugation of oligonucleotides to peptides have hitherto proved elusive. The native ligation strategy we have developed has many attractions in this respect. First, the reagents used for functionalization of peptide and oligonucleotide moieties are stable solids that are prepared in each case in two steps from readily accessible materials. Second, the reagents are used in standard protocols of solid-phase

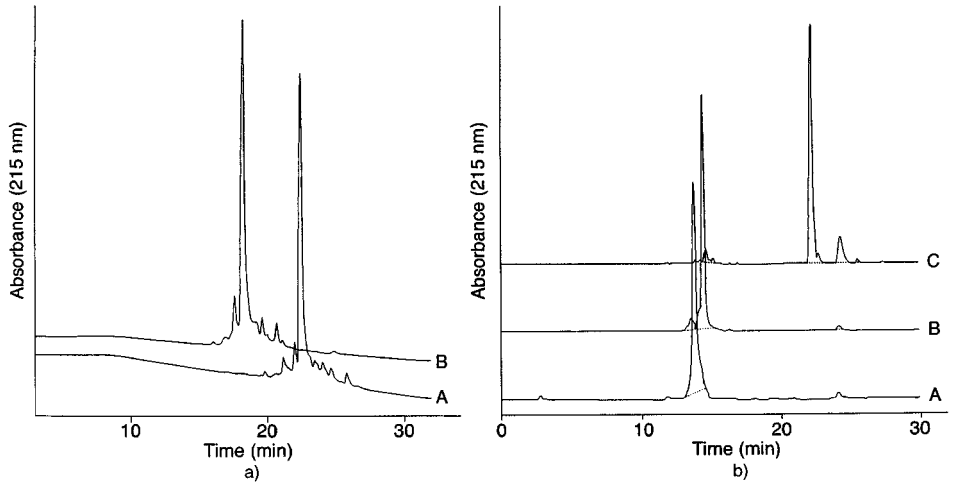


Figure 1. (a) Analytical reversed-phase HPLC of crude (A) H-PQIK(Tfa)IWFPNRRK(Tfa)PFK(Tfa)K(Tfa)-NH_2 and (B) **P5** thioester (Table 1, entry 5), as obtained after acidolytic cleavage. HPLC conditions as listed in the Experimental Section, General Procedures. (b) Analytical HPLC of (cysteinylamino)cyclohexyl-modified oligonucleotides: (A) H-Cys-GCT CCC AGG CTC AAA **ODN2** (Table 2, entry 7), (B) H-Cys(StBu) **ODN2** (Table 2, entry 5), and (C) H-Cys(Trt)-TTT TT **ODN1** (Table 2, entry 2). HPLC conditions (a), as listed in the Experimental Section.

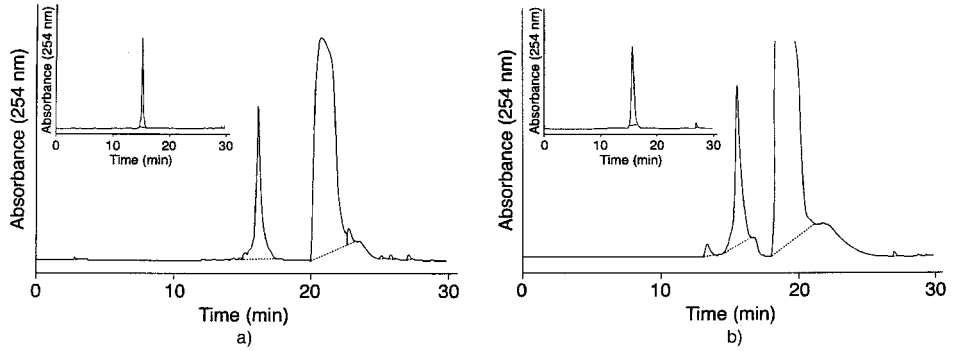


Figure 2. (a) Reversed-phase HPLC of **ODN1**−**P1** conjugate (Table 3, entry 1), reaction mixture after 24 h, (conditions b). Inset: purified conjugate (conditions a). (b) Reversed-phase HPLC of **ODN2**−**P1** conjugate (Table 3, entry 2), reaction mixture after 24 h (conditions b). Inset: purified conjugate (conditions b). Note that the most prominent peak eluting at around 20 min is thiophenol.

synthesis and couple to their respective peptide or oligonucleotide partners in high yield. Third, assuming the peptide and oligonucleotide fragments are themselves assembled efficiently, the functionalized oligonucleotides and peptides may be used in unpurified form for conjugation without the need for further manipulation following deprotection and release from their respective solid supports.

We have shown some examples of conjugation reactions using peptides and oligonucleotides of mixed composition and of length and type appropriate for biological studies. In these examples, we have begun to learn about the requirements for efficient conjugation. First, the rate of conjugation appears to be dependent on the concentrations of the two components. Reactions carried out with 2.5 mM peptide **P1** and 0.5 mM oligonucleotide gave high yields within a 24 h period either in the presence or in the absence of a denaturing agent (7 M urea). By contrast, reactions carried out at 10-fold lower concentrations (e.g., with peptides **P2** and **P3**) still showed some starting oligonucleotide even after 48 h and some mixed disulfide side-products were also in evidence in some cases in addition to the desired product. Nevertheless the conjugate was the major product in all examples shown. We are currently exploring the possibility of addition of a more reducing thiol during ligation, such as benzyl mercaptan that was recently suggested,[43] or addition of another reducing agent after ligation is complete. A side-reaction resulting from intramolecular cyclization of the succinyl functionality on the peptide was observed in place of conjugation in the case of N-terminal glycine, but no cyclization was detected in the case of a peptide containing N-terminal alanine. Use of N-terminal proline or sarcosine peptides obviates any possibility of cyclization. We have also learnt that in cases where conjugation may be slow, prior TCEP reduction of the oligonucleotide component at pH 6.5 to remove the *tert*-butylsulfenyl protecting group may also be helpful.

Further experimentation will be necessary to optimize the solvent composition and reaction conditions for conjugation, especially in the case of longer peptides that may have secondary structure, but in the case of hydrophobic peptide segments the addition of DMF has helped to maintain solubility. But there is already a sufficient number of examples to suggest that in principle a very wide range of peptides should be able to be conjugated to oligonucleotides by the native ligation technique and there should be very few restrictions to peptide sequence. It should be noted that internal cysteine residues in a peptide do not interfere with native ligation reactions.[37] Our ligation method has advantages over that described recently for RNA-peptide conjugation by McPherson et

al.[41] Our method is in principle suitable for conjugation of peptides containing thioesters at the C- or N-terminus and utilizes chemically synthesized 5′-cysteine-substituted oligonucleotides obtained by standard phosphoramidite methods. By contrast, the method of McPherson et al is restricted to N-terminal cysteine-containing peptides and to transcribed RNA which is functionalized with a 5′-thiophosphate and then alkylated to produce a 5′-thioester. Further, reagent **2** could be used to prepare thioesters from other amino-functionalized biomolecules.

In the future, we plan to investigate the conjugation of peptides to 3′-cysteine-functionalized oligonucleotides, to oligonucleotides bound to solid supports, as well as the use in conjugation reactions of C-terminal peptide thioesters prepared by new Fmoc synthetic routes.[45,48] We also hope to extend the conjugation chemistry to other oligonucleotide analogues which are more commonly used for antisense experiments, such as oligodeoxynucleotide phosphorothioates and 2′-*O*-alkyl oligoribonucleotides, that are more resistant to attack by nucleases. There should be no impediment in principle to such conjugations using native ligation technology. In applications of such conjugates in cellular uptake studies, the presence of a free thiol in the cysteine residue remaining at the join between peptide and oligonucleotide parts could be useful for attachment of a fluorescent or other reporter group. In conclusion, we believe the method should be generally applicable for the development of procedures for efficient peptide−oligonucleotide conjugation.

Experimental Section

General Procedures. Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Rink amide NovaGel HL and Fmoc-PAL-PEG-PS resins were purchased from Novabiochem and PE Biosystems, respectively. Fmoc-amino acids and derivatives were purchased from Novabiochem, Millipore and PE Biosystems. DMF (Fisher) was distilled in vacuo and used shortly after. Dichloromethane and acetonitrile (Fisher) were refluxed over CaH_2 followed by distillation. Basic solvents were purchased from Fisher. Standard peptide synthesis reagents were purchased from PE Biosystems (HATU, HOAt, DIEA) and Romil (UK) (piperidine, trifluoroacetic acid). All other fine chemicals were supplied by Aldrich and Fluka. Thin-layer chromatography was carried out on Merck 60 F_{254} aluminum-coated silica gel plates. TLC elution systems: (a) EtOAc−CH_2Cl_2−AcOH (10:85:5 v/v/v) (system A); (b) EtOAc−hexane (1:1 v/v) (system B); (c) MeOH−$CHCl_3$−AcOH (2:93:5 v/v/v) (system C); (d) EtOAc−hexane−NEt_3 (35:60:5 v/v/v) (system D). Spots were visualized by UV irradiation with a 254 nm lamp. Column chromatography was carried out using Macherey-Nagel 60 230−400 mesh silica gel. All ^1H and ^{31}P NMR spectra were recorded at 300 MHz. MALDI-TOF mass spectra were recorded on a Voyager-DE workstation (PE Biosystems). Matrixes used for preparing MALDI-TOF samples were the following: (a) α-cyano-4-hydroxycinnamic acid, 10 mg mL^{-1} in acetonitrile-3% aqueous TFA (1:1 v/v) for all peptides, (b) 2,6-dihydroxyacetophenone, 20 mg mL^{-1}, and diammonium hydrogen citrate, 40 mg mL^{-1}, in 50% aqueous methanol for all oligonucleotides, conjugates and phosphoramidites, and (c) 2,5-dihydroxybenzoic acid, 10 mg mL^{-1} in methanol for all other low molecular weight compounds. Elemental analyses were carried out by The Chemical Laboratory, University of Cambridge, UK. Analytical HPLC of peptides was carried out with dual wavelenth (215 and 230 nm) UV detection using a Vydac RP-C8 column (4.6 × 250 mm). Preparative peptide chromatography was carried out using a Vydac RP-C8 column (25 × 300 mm) and 215 nm UV detection. Oligonucleotide and conjugate analytical and semipreparative HPLC was carried out using a Phenomenex RP-C18 column (4.6 × 250 mm) and dual wavelength (218 and 254 nm) UV detection.

General Methods for Solid-Phase Peptide Synthesis. Solid-phase peptide synthesis was carried out by the solid-phase Fmoc-*tert*-butyl procedures[49] on a PE Biosystems Pioneer peptide synthesis system on a 0.1 mmol scale using HATU/DIEA in situ activation protocols[50] and either a Rink NovaGel HL or a PAL-PEG-PS solid support. After removal of the last *N*-α-Fmoc group, pentafluorophenyl *S*-benzyl thiosuccinate **1** was manually coupled to the last amino acid (4.5 equiv of **1**, 1 equiv of HOAt in 2 mL of DMF) for 4 h at room temperature. Alternatively, *S*-benzyl thiosuccinic acid can be coupled using the same HATU protocol as for standard peptide chain assembly. Then the resin was washed with DMF (5 × 5 mL), MeOH (3 × 5 mL), and diethyl ether (2 × 5 mL), and dried. Cleavage from the support and deprotection of the side chain protecting groups was carried out in TFA−benzyl mercaptan−phenol−water (90:5:2.5:2.5 v/v/v/v) mixture for 1−6 h at room temperature depending on N^G-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) arginine content. The filtrate was flushed by a stream of nitrogen to remove most of the TFA and the peptide thioesters were precipitated by addition of cold (−20 °C) diethyl ether, washed thrice with diethyl ether, and dried in vacuo. The solid was redissolved in 0.1% TFA with a variable proportion of acetonitrile (10−30%) to solubilize hydrophobic peptide thioesters, to ca. 3−5 mg mL^{-1} concentration, and subjected to HPLC analysis using a gradient of acetonitrile in 0.1% aqueous TFA from 10 to 90% in 30 min. If needed, peptide thioesters were purified by preparative HPLC. Appropriate fractions were pooled, lyophilized, and analyzed by MALDI-TOF MS. Analytical data of the synthesized peptide thioesters are given in Table 1.

General Methods for Solid-Phase Oligonucleotide Synthesis. Assembly of oligonucleotides was carried out by the standard 2-cyanoethyl phosphoramidite method[51] on long chain alkylamine controlled pore glass (LCAA-CPG) support (Glen Research via Cambio). All oligonucleotides were synthesized on a 1 μmol scale using an ABI 380B DNA/RNA Synthesizer. Protected 2′-deoxyribonucleoside phosphoramidites were obtained from Cruachem (Scotland). After the last dimethoxytrityl group removal, modified phosphoramidite **5** or **6** (0.15 M solution in dry acetonitrile) was coupled to the support-bound oligonucleotide using an extended coupling protocol (10 min) to ensure complete coupling. After the usual iodine-water oxidation, the support was manually flushed with 20% piperidine solution in DMF for 10 min to remove Fmoc groups, washed with 10 mL of DMF, 10 mL of acetonitrile and briefly air-dried. The glass beads were then treated with 0.5 mL of 30% aqueous ammonia solution at room temperature for 2 h to cleave the oligonucleotide from the support, washed with an additional 0.5 mL of concentrated ammonia solution, and the filtrate was then transferred to a screw-capped polypropylene tube and heated at 55 °C for 16 h to completely deprotect oligonucleotides at the nucleobase and phosphate residues. After cooling and evaporating most of the solution under a stream of nitrogen, 1 mL of deionized water was added and the solution was evaporated on a SpeedVac vacuum concentrator to dryness, product redissolved in deionized water, and the quantity of the oligonucleotide was then assessed by checking the absorbance at 260 nm on a Perkin-Elmer Lambda 2 UV−vis spectrophotometer. Purity of oligonucleotides was established by analytical HPLC using gradients of acetonitrile 0−2%, 5 min, 2−40% 20 min, 40−100% 25 min in either 0.1 M ammonium acetate buffer, pH 7.0 (a), or 0.1M triethylammonium acetate buffer, pH 7.0 (b). Molecular

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masses were confirmed by MALDI-TOF mass spectrometry (positive ion mode). Analytical data are summarized in Table 2.

General Methods for Peptide−Oligonucleotide Conjugation by Native Ligation. The following oligonucleotide sequences were used for model conjugation studies: TTT TT (**ODN1**), GCT CCC AGG CTC AAA (**ODN2**), and AGC TCC CAG GCT CAA (**ODN3**). Three representative peptide N-terminal thioesters were chosen for conjugation: HIV-1 Tat protein C-terminal 14-mer PTSQSRGDPTGPKE (**P1**), a hydrophobic pentapeptide Sar-Leu-Gly-Ile-Gly (**P2**), and HIV-1 Rev protein nuclear export signal (NES) decamer ALPPLERLTL (**P3**). Four sets of conditions in generally 1 mL reaction volumes were explored: (a) 0.5 mM ODN, 5 equiv of peptide, 0.1 M TCEP, 2% PhSH (v/v), 7 M urea, 0.1 M sodium phosphate, pH 7.5, 24 h, room temperature (conditions **A**); (b) 0.5 mM ODN, 5 equiv of peptide, 0.1 M TCEP, 2% PhSH (v/v), 25% DMF (v/v) in 0.1 M sodium phosphate, pH 7.5, 24 h, room temperature (conditions **B**); (c) 0.05 mM ODN, 10 equiv of peptide, 0.1 M TCEP, titrated to pH 6.5 by addition of 20% sodium hydroxide solution, 2% PhSH (v/v), 25% acetonitrile, 48 h, room temperature (conditions **C**); (d) 0.1 mM ODN, pretreated with 0.2 M TCEP, pH 6.5, for 3 h at room temperature, then 10 equiv, peptide in an equal volume of 50% aqueous DMF was added together with PhSH (2% v/v final) and kept at room temperature for 48 h (conditions **D**). Reaction mixtures were analyzed by reversed phase HPLC and products were separated by semipreparative RP-HPLC. Fractions containing products were lyophilized twice from deionized water and analyzed by MALDI-TOF. Analytical data for conjugates synthesized are given in Table 3.

***S*-Benzyl Thiosuccinic Acid (1).** Benzyl mercaptan (2.595 mL, 22 mmol) was added under nitrogen to a stirred solution of succinic anhydride (2.00 g, 20 mmol) and 4-(dimethylamino)-pyridine (122.2 mg, 1 mmol) in 25 mL of anhydrous acetonitrile/pyridine (9:1 v/v). Stirring was continued at room temperature for 3 h and the mixture was evaporated to near dryness. The product was dissolved in 30 mL of aqueous sodium bicarbonate solution, pH 8.5, and extracted twice with 10 mL of diethyl ether. The aqueous phase was then cooled in an ice bath and acidified with 5 N hydrochloric acid to pH 2. The white precipitate was filtered off, washed with cold 0.1M HCl solution, followed by ice-cold water, and dried in a vacuum desiccator over phosphorus pentoxide overnight. Yield of white powder 3.6 g (80%). TLC (A): R_f 0.76. ^{1}H NMR ($CDCl_3$): δ 2.74 (t, 2H, J = 6.6 Hz), 2.92 (t, 2H, J = 6.8 Hz), 4.16 (s, 2H), 7.27 (m, 5H). MALDI-TOF MS: $[M + Na]^+$ 247.6 (247.3 calcd), $[M + K]^+$ 263.4 (263.2 calcd). Anal. Calcd for $C_{11}H_{12}O_3S$: C, 58.91; H, 5.39. Found: C, 58.69; H, 5.41.

Pentafluorophenyl *S*-Benzyl Thiosuccinate (2). A solution of dicyclohexyl carbodiimide (2.27 g, 11 mmol) in 15 mL of dichloromethane was added dropwise to a stirred and cooled (ice bath) solution of *S*-benzyl thiosuccinic acid (2.24 g, 10 mmol) and pentafluorophenol (2.12 g, 11.5 mmol) in 25 mL of dichloromethane. The reaction mixture was stirred for 0.5 h in an ice bath and then allowed to warm slowly to room temperature, stirred for 4 h and left overnight in a refrigerator. Dicyclohexylurea precipitate was filtered off and the remaining solution concentrated in vacuo, redissolved in a minimal volume of ethyl acetate, the solution filtered again to remove particulates, and hexane was added. After standing overnight in a freezer, crystals were filtered off, washed with cold ethyl acetate and hexane mixture (1:9 v/v) and dried in vacuo overnight. Yield of white needles 3.44 g (88%). After evaporation of mother liquor and further treatment with hexane, an additional 0.18 g of the title compound was obtained. Total yield of two crops 3.62 g (92%). TLC (B): R_f 0.84. ^{1}H NMR (DMSO-d_6): δ 3.08 (s, 4H), 4.16 (s, 2H), 7.26 (m, 5H). MALDI-TOF MS: $[M + H]^+$ 393.4 (391.3 calcd). Anal. Calcd for $C_{17}H_{11}F_5O_3S$: C, 52.31; H, 2.84. Found: C, 51.50; H, 2.85.

***N*-α-Fmoc-*S*-*tert*-butylsulfenyl-L-cysteine 4-Hydroxy-*trans*-cyclohexylamide (3).** To a slurry of *trans*-4-aminocyclohexanol hydrochloride (0.3 g, 2 mmol), *N*-α-Fmoc-*S*-*tert*-butylsulfenyl-L-cysteine pentafluorophenyl ester (1.2 g, 2 mmol), and 1-hydroxybenzotriazole (2 mmol, 270.3 mg) in 20 mL of anhydrous DMF was added triethylamine (0.45 mL, 3.1 mmol), and the resulting solution was stirred at room temperature for 3 h until TLC showed complete reaction. The reaction mixture was evaporated to dryness and the white residue transferred to a sintered glass filter, washed successively with small amounts of DMF, ethanol, and diethyl ether, and dried in vacuo. Yield of white powder: 0.87 g (82%). TLC (C): R_f 0.35. ^{1}H NMR (DMSO-d_6): δ 1.18 (m, 4H), 1.27 (s, 9H), 1.69 (m, 2H), 1.77 (m, 2H), 2.95 (m, 2H), 3.45 (m, 2H), 4.24 (m, 4H), 7.30 (t, 2H, J = 7.4 Hz), 7.41 (t, 2H, J = 7.4 Hz), 7.61 (d, 1H, J = 8.5 Hz), 7.72 (d, 2H, J = 7.3 Hz), 7.86 (m, 3H). MALDI-TOF MS: $[M + H]^+$ 528.9 (529.7 calcd), $[M + Na]^+$ 550.8 (551.7 calcd), $[M + K]^+$ 566.6 (567.7 calcd). Anal. Calcd for $C_{28}H_{36}N_2O_4S_2$: C, 63.61; H, 6.86; N, 5.30. Found: C, 63.85; H, 6.84; N, 5.28.

***N*-α-Fmoc-*S*-trityl-L-cysteine 4-hydroxy-*trans*-cyclohexylamide (4).** To a slurry of *trans*-4-aminocyclohexanol hydrochloride (0.32 g, 2.1 mmol) and *N*-α-Fmoc-*S*-trityl-L-cysteine pentafluorophenyl ester (1.5 g, 2 mmol) in 25 mL of anhydrous DMF was added triethylamine (0.31 mL, 2.2 mmol), and the resulting solution was stirred at room temperature for 3 h until TLC showed complete reaction. The reaction mixture was evaporated to dryness, redissolved in ethyl acetate and washed successively with ice-cold 5 wt % citric acid solution, water, 5% sodium bicarbonate solution, and brine, dried over sodium sulfate, and evaporated to a light brown foam. The residue was chromatographed on a silica gel column eluted by 5−15% ethyl acetate in hexane + 0.5% triethylamine. Appropriate fractions were pooled and evaporated to give 1.31 g (95%) of the title product as a white foam. TLC (C): R_f 0.42. ^{1}H NMR (DMSO-d_6): δ 1.17 (m, 4H), 1.65 (m, 2H), 1.75 (m, 2H), 2.29 (m, 2H), 3.36 (m, 2H+water), 4.01 (q, 1H, J = 7.0 Hz), 4.22 (m, 3H), 4.50 (d, 1H, J = 4.3 Hz), 7.28 (m, 17H), 7.39 (t, 2H, J = 7.4 Hz), 7.57 (d, 1H, J = 8.7 Hz), 7.66 (d, 1H, J = 7.7 Hz), 7.72 (d, 2H, J = 7.3 Hz), 7.87 (d, 2H, J = 7.5 Hz). MALDI-TOF MS: $[M + Na]^+$ 704.1 (705.9 calcd), $[M + K]^+$ 720.0 (721.8 calcd). Anal. Calcd for $C_{43}H_{42}N_2O_4S$: C, 75.63; H, 6.20; N, 4.10. Found: C, 75.33; H, 6.10; N, 4.11.

***O*-*trans*-4-(*N*-α-Fmoc-*S*-*tert*-butylsulfenyl-L-cysteinyl)-aminocyclohexyl *O*-2-Cyanoethyl-*N*,*N*-diisopropylphosphoramidite (5).** To a chilled (ice bath) solution of **3** (0.83 g, 1.578 mmol) in 15 mL of anhydrous dichloromethane containing 3 equiv (0.79 mL) of diisopropylethylamine was added dropwise via syringe and under nitrogen 2-cyanoethoxy-*N*,*N*-diisopropylaminochlorophosphine (1.5 equiv, 0.529 mL). After 1 h of stirring on ice, the mixture was allowed to warm gradually to room temperature and stirring was continued for 2 h. The reaction mixture was quenched with 0.1 mL of methanol and evaporated to dryness. The product was taken up in ethyl acetate, washed with saturated sodium bicarbonate solution and brine, dried over anhydrous sodium sulfate and evaporated to a small volume. The product was chromatographed on a silica gel column eluted with 15−40% ethyl acetate in hexane + 2% triethylamine. Appropriate fractions were pooled and evaporated to dryness. Yield of title product: 0.76 g (66%). TLC (D): R_f 0.53. ^{1}H NMR (CD_3CN): δ 1.16 (d, 6H, J = 0.9 Hz), 1.18 (d, 6H, J = 1.0 Hz), 1.27 (m, 1H), 1.32 (s, 9H), 1.45 (m, 1H), 1.87 (m, 2H), 1.95 (q, 2H, J = 2.5 Hz), 2.01 (m, 1H), 2.64 (t, 2H, J = 5.9 Hz), 2.95 (m, 1H), 3.12 (m, 1H), 3.62 (m, 3H), 3.75 (m, 2H), 4.32 (m, 3H), 6.07 (d, 1H, J = 8.6 Hz), 6.59 (d, 1H, J = 7.8 Hz), 7.34 (t, 2H, J = 7.5 Hz), 7.43 (t, 2H, J = 7.4 Hz), 7.68 (d, 2H, J = 7.3 Hz), 7.85 (d, 2H, J = 7.5 Hz). ^{31}P NMR (CD_3CN): δ 146.51 ppm. MALDI-TOF MS: $[M + H]^+$ 729.4 (730.0 calcd).

***O*-*trans*-4-(*N*-α-Fmoc-*S*-trityl-L-cysteinyl)aminocyclohexyl *O*-2-Cyanoethyl-*N*,*N*-diisopropylphosphoramidite (6).** To a solution of **4** (0.24 g, 0.34 mmol) in 10 mL of anhydrous dichloromethane containing 75 mg (1.5 equiv) of diisopropylammonium tetrazolide was added 2-cyanoethoxy-*N*,*N*,*N*′,*N*′-tetraisopropyl phosphorodiamidite (0.13 mL, 0.39 mmol), and the mixture was stirred for 6 h at room temperature, until TLC revealed complete reaction. Dichloromethane was then removed by evaporation and the product was taken up in ethyl acetate, washed with 5% sodium bicarbonate

solution and brine, dried over anhydrous sodium sulfate and evaporated to a small volume. The product was chromatographed on a silica gel column eluted with 10−35% ethyl acetate in hexane + 2% triethylamine. Appropriate fractions were pooled and evaporated to dryness. Yield of title product 1.30 g (95%). TLC (D): R_f 0.78. ^{1}H NMR (DMSO-d_6): δ 1.11 (d, 6H, J = 1.6 Hz), 1.13 (d, 6H, J = 1.4 Hz), 1.19 (m, 8H), 1.35 (m, 2H), 1.69 (m, 2H), 1.86 (m, 2H), 2.28 (m, 2H), 2.76 (t, 2H, J = 5.8 Hz), 3.48 (m, 4H), 3.67 (m, 3H), 4.02 (q, 1H, J = 7.1 Hz), 4.22 (m, 3H), 7.29 (m, 17H), 7.39 (t, 2H, J = 7.5 Hz), 7.59 (d, 1H, J = 8.6 Hz), 7.72 (d, 2H, J = 6.9 Hz), 7.88 (d, 2H, J = 7.5 Hz). ^{31}P NMR (CD_3CN) δ 146.50 ppm. MALDI-TOF MS: $[M + H]^+$ 922.0 (922.2 calcd).

Acknowledgment. We thank Richard Grenfell and Donna Williams for oligonucleotide assemblies and Robert C. Sheppard and David Owen for valuable advice on peptide synthesis.

Supporting Information Available: NMR spectra of intermediates and examples of HPLC traces and MALDI-TOF spectra of peptide thioesters, cysteine oligonucleotides and conjugates. This material is available free of charge via the Internet at http://pubs.acs.org.

JO000214Z