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Synthesis, Purification and Characterization of Two Peptide-Oligonucleotide Conjugates as Potential Artificial Nucleases

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Abstract: Two peptide-oligonucleotide conjugates **10a** and **10b**, containing as their peptide moiety the active site mimic of ribonuclease A (HGH motif) and the Cu (II) complexing metallopeptide (GGH motif) respectively, have been synthesized by original on-line solid phase synthesis using pentafluorophenyl active esters and Boc-His(Tos)-OH. Mild basic conditions for the final deprotection and reversed-phase purification afforded the pure hybrid molecules in good yields. The conjugates have been characterized by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

INTRODUCTION

The recent development of chemical oligonucleotide synthesis allows the generation of conjugates exhibiting specific chemical activities. Sequence-specific hydrolytic nucleases based on inorganic complexes or diamines conjugated to DNA that successfully cleave RNA have been published[1]. Peptide-oligonucleotide conjugates have attracted great interest in order to broaden the spectrum of oligonucleotide properties and represent a new chemical way to increase the activity of synthetic oligonucleotides used as inhibitors of gene expression. Examples include the use of peptide signal sequences[2], hydrophobic groups such as tryptophane[3], cationic poly-L-Lys to increase cellular uptake[4] or to provide non-radioactive probes for PCR studies[5], cationic poly-L-Arg[6] and α-helical peptides[7]. Another perspective for peptide-oligonucleotide hybrid molecules is the development of specific artificial nucleases. Enzymes able to hydrolyse selectively single stranded DNA and RNA can be anchored to oligonucleotide moiety and provide highly efficient and selective nucleases[8]. However, for *in vivo* applications, it might be preferable to use nuclease mimics with low molecular weights such as the active site of ribonuclease A or small metallopeptides. The histidyl-glycyl-histidyl (HGH) motif has been suggested to mimick the active site of RNase A on the basis of the cleavage mechanism of this enzyme[9]. Tung et al[10] have recently reported that this mimic tripeptide is able to cleave RNA under physiological conditions. They also observed similar activities with a glycyl-glycyl-histidyl (GGH) motif, thus confirming that an imidazole group is not required at the N-termini of the peptide sequence. The hydrolytic mechanism involves both the abstraction of a proton from the 2'-hydroxyl group by the imidazole nitrogen

of the third residue and the stabilization of the pentacoordinated cyclic transition state by the protonated α-amino group of the first residue. Other synthetic RNA-cleaving molecules imitating the catalytic site of RNase A have also been elaborated[11] and confirmed these data. The tripeptide GGH has also been demonstrated to be a Cu (II) and Ni (II) chelator[12]. This motif has been conjugated to DNA binding Hin recombinase[13], to trifluoroperazine[14], to netropsine[15] and to the DNA binding domain of transcription factor SP1 (with three zinc fingers)[16]. These conjugates provide efficient sequence-specific DNA-cleaving molecules in the presence of reducing agents after Cu (II) or Ni (II) complexation. However, the incorporation of GGH sequences has been limited to the amino termini of peptide sequences. Shullenberger et al[17] have developed a strategy to insert a tripeptide sequence (δ)-Orn-Gly-His in a peptide chain via the δ-amino group of Orn. This design leaves the α-amino group of Orn free to participate in Cu (II) complexation. Along this strategy, we decided to synthesize artificial nucleases containing HGH or GGH as the peptide moiety and a 12-mer complementary to a Ha-ras mRNA sequence as the oligonucleotide moiety.

Recently, we described a new method for the preparation of oligonucleotide-peptide hybrid molecules by means of automated synthesis[7a]. In the present article, we report an extension of the strategy used emphasizing a) the easy incorporation of His residue in conjugates, b) the very good yields obtained after one step deprotection and reversed-phase purification and c) the complete characterization of these hybrid molecules by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS).

RESULTS AND DISCUSSION

Synthesis

The on-line solid phase synthesis of oligonucleotide-peptide hybrids requires appropriate amino acid side chain protections. Although both Boc and Fmoc strategies are compatible with solid phase synthesis of conjugates on silica supports, the choice of side chain protecting groups must be carefully considered. Each amino acid protection must be chosen as to allow i) the efficient elongation of the growing chain and ii) the easy removal of the protecting group under the very mild acidic or basic conditions used to safely deprotect oligonucleotide bases. When possible, the use of commercially available amino acid derivatives is preferred.

In conventional solid phase peptide synthesis, the imidazole ring of histidine is usually protected with the Π-Bom group. The Π-Bom group seemed suitable since it can be removed under mild conditions by catalytic transfer hydrogenation which does not affect the oligonucleotide moiety[18]. However, the Boc-His(Π-Bom) deprotection with palladium black and 1,4-cyclohexadiene did not afford the desired conjugates in our hands, due to strong adsorption of the conjugate on the catalyst. The Fmoc-His(Boc) derivative used by Bashkin et al[19] requires a TFA treatment which is harmful for the purine bases. The Boc-His(Fmoc)-OH seems to be significantly unstable during a multistep synthesis. The dinitrophenyl protecting group (Dnp) is usually removed by mercaptans. It can also be easily cleaved by NH_4OH[20] or sodium hydroxide treatments. However,

the Dnp group leads to yellow contamined products after deprotection. We found that the most satisfactory commercially available protected histidine derivative was Boc-His(Tos)-OH. We were aware that His(Tos) may racemize during the coupling steps especially when using DCC/DMAP as condensing agent. Replacing DMAP by HOBt, a procedure known to reduce the extent of racemization, was not possible because HOBt is known to cleave the N^{im}-Tosyl group[21]. In order to maintain the racemization level as low as possible, we used a catalytic amount of DMAP (0.1 equivalent). No systematic check for racemization was undertaken on the final product. The tosyl group is easily removed by NH_4OH or NaOH solutions at low concentrations without side-reaction or colored contaminants. The deprotection step required a treatment by 0.1 M NaOH for about 24 hours. Under these conditions, racemization was expected to be limited because alkaline saponifications are generally run under drastic conditions without large extents of racemization. For instance, a treatment by 0.5 M NaOH for 4 hours at room temperature affords only about 2 % of the D enantiomers[22].

Conventional coupling steps in peptide synthesis utilize DCC/HOBt activation of the aminoacid derivatives. As already mentioned, HOBt cleaves the tosyl group of histidine. To overcome this difficulty, we coupled aminoacid pentafluorophenyl active esters in the presence of triethylamine. These powerful acylating agents rapidly react with polymer-bond nucleophiles[23] and are now currently used in SPPS. Several of the pentafluorophenyl esters derivatives are difficult to prepare in a pure state[24]. However, the crude ester can be used in the coupling reaction and give the corresponding optically pure peptides in high yield. Triethylamine was used because it induces less racemization than other tertiary amines[25].

For the HGH sequence synthesis, we used Boc-His(Tos)-OH, Boc-Gly-OPfp, Boc-His(Tos)-OPfp and $DMTrO(CH_2)_9CO_2Pfp$ as spacer arm between the peptide and oligonucleotide moieties. To mimick the N-terminal H-Gly-Gly-His motif, we inserted the Lys-Gly-His sequence using Fmoc-Lys(Boc)-OPfp derivative. After Boc N^{ε}-deprotection with TFA, the chain was elongated at the ε-amino group of lysine. On the final deprotected product, the free α-amino group of lysine can served as proton acceptor or can participate in the Cu (II) complexation as does the free N^{α}-amino function of glycine in the GGH motif.

The on-line synthesis of oligonucleotide-peptide hybrid molecules was run on a silica support[7a]. The Controlled Pore Glass (CPG) support **2** appeared to be the best candidate to obtain the desired products in good yields. In a previous report, we stated that both CPG and Fractosil supports were suitable. Recent control syntheses (unpublished results) of conjugates containing the same deoxyribonucleotide heterosequence on both CPG and Fractosil support revealed that CPG was more efficient since it afforded higher yields with lower loads. De la Torre *et al.*[2b] reported peptide sequence deletions when using CPG. We did not observe such deletions for the simple tripeptides described in this study and for the leucine and lysine dodecapeptides previously published[7a]. The side-reactions observed by De la Torre *et al.* may depend on the nature of the peptide sequence.

In the strategy outlined on Figure 1, the support was first derivatized with $DMTrO(CH_2)_9CO_2Pfp$ **1** to introduce a spacer arm ended by a terminal hydroxyl group. Only 40 eq. of pentafluorophenyl ester were

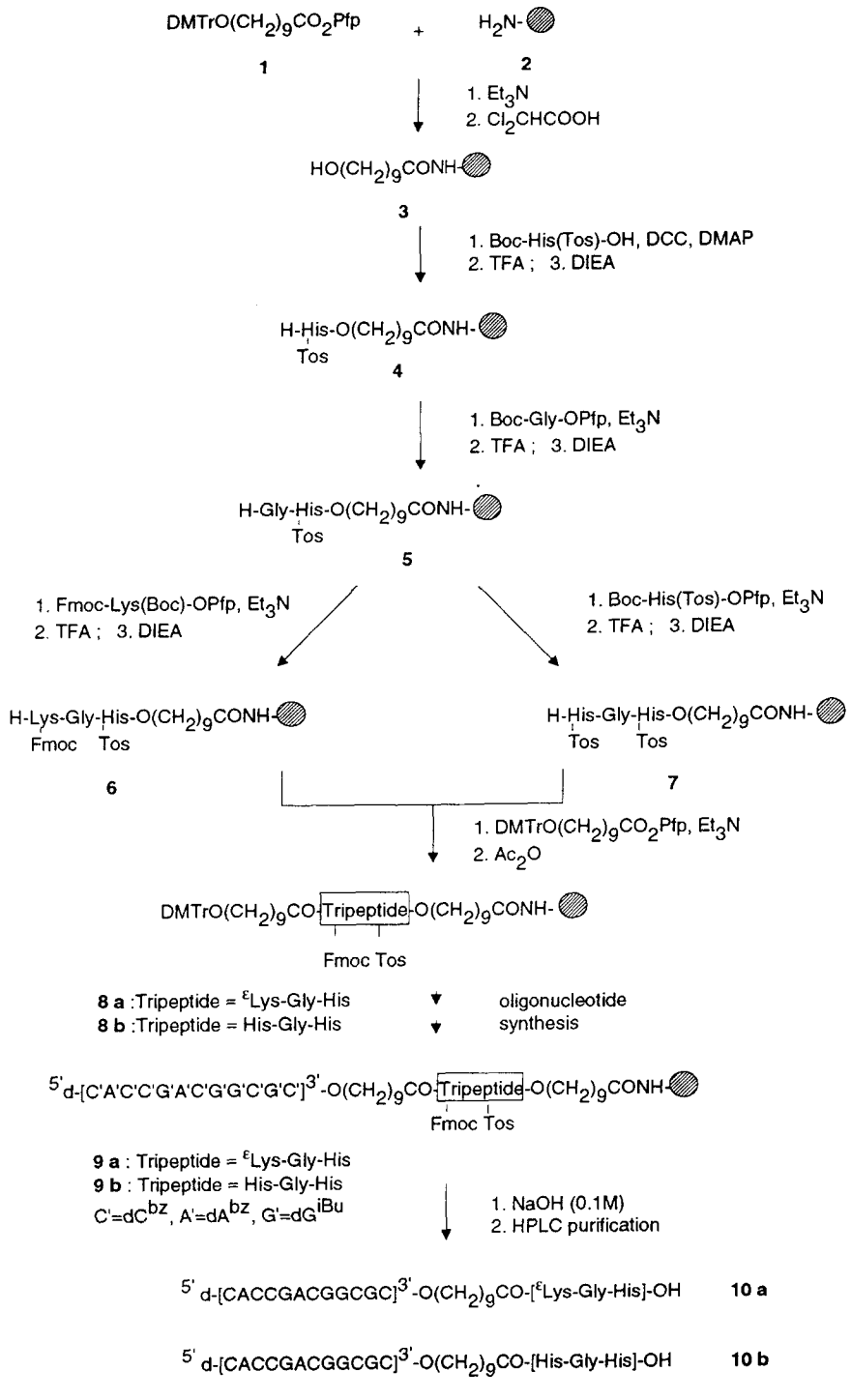

Figure 1: Synthesis of peptide-oligonucleotide hybrid molecules **10a** and **10b**.

required to achieve the coupling reaction while the corresponding *p*-nitrophenyl ester required 100-150 eq. After attachment of Boc-His(Tos) in the presence of DCC and DMAP, subsequent couplings were carried out using crude pentafluorophenyl active esters. Each aminoacid derivative was coupled using 40 eq. of active ester in the presence of triethylamine. Acetylation was run after each coupling step to cap the unreacted amino groups. The supported peptides were then treated with 40 eq. of the spacer reagent **1** and acetylated. The substrates were transferred to an automated DNA synthesizer, Pharmacia Gene Assembler, and oligonucleotide chain elongation was run via cyanoethyl phosphoramidite chemistry[26].

Deprotection and Purification

The deprotection of the conjugates was first attempted with ethanolamine in absolute ethanol for 30 hours at 60°C[7a]. This treatment produces a peptide C-terminal ethanolamide while concentrated aqueous ammonia generates an undesirable mixture of C-terminal amide and carboxylate[27]. The yields obtained with ethanolamine and deoxyribonucleotide heterosequences were very low (< 2%). To optimize the deprotection step, sodium hydroxide was tested at room temperature and afforded crude conjugates **10a** and **10b** in high yields without side-reactions. The ester linkage which anchors the conjugates to the support is cleaved in 2 hours instead of 24 hours with ethanolamine or ammonia. Sodium hydroxide simultaneously cleaves lysine Fmoc, histidine tosyl and phosphate cyanoethyl protecting group in one hour. However, complete nucleic base deprotection requires 24 hours. Analysis of the crude product (see for instance **10a** on Figure 2) by reversed-phase HPLC after sodium hydroxide deprotection followed by acetic acid neutralization gave the desired product as the major peak. The products **10a** and **10b** were purified by preparative HPLC and quantified by UV spectrophotometry.

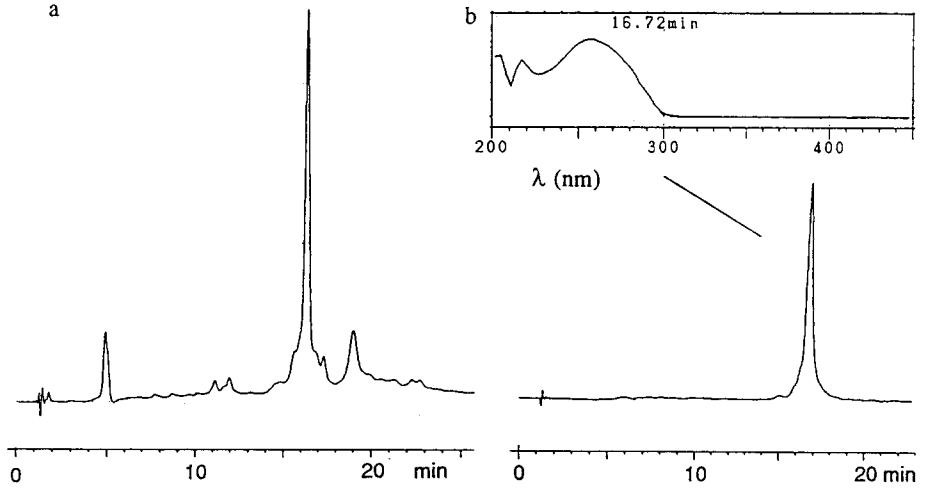

Figure 2 : HPLC profiles of (a) crude peptide-oligonucleotide conjugate **10a** and (b) purified **10a** with the corresponding absorption spectra recorded between λ = 200 nm and λ = 450 nm. See experimental section for analysis conditions.

Characterization

Although it is possible to characterize the peptide moiety by amino acid analysis or mass spectrometry and the oligonucleotide part by digestion with nucleases, it is necessary to have a direct and unambiguous method to ascertain the chemical integrity of the peptide-oligonucleotide conjugates.

Recently, Pieles *et al.*[28] have proposed the use of MALDI-TOF MS to characterize natural and modified oligonucleotides. They demonstrated that a non carboxylic acid matrix, 2,4,6-trihydroxy acetophenone used in combination with diammonium salts was highly efficient for the desorption of oligonucleotides. We used this technique to determine the molecular-ion weight of the peptide-oligonucleotide hybrid molecules. Besides a strong signal of deprotonated molecular ion $[M-H]^-$, signals of $[M-2H]^{2-}$ and $[2M-H]^-$ were observed in the spectrum with lower intensity (Figure 3). The molecular weights of compounds **10a** and **10b** have been determined by using a combination of diammonium-L-tartrate and 2,4,6-trihydroxy acetophenone as matrix with an accuracy of ± 2 mass units (deviation < 0.05 %) without using an internal standard. The instrument was calibrated with a sample of $d(T)_{12}$ which exhibits two distinct peaks ($[M-H]^-$: m/z = 3585.4 and $[2M-H]^-$: m/z = 7170.8). Oligothymidylic acids were chosen as mass standards because of their high stability and homogeneity. As already reported for oligonucleotides[29], peptide-oligonucleotide conjugates **10a** and **10b** yield stronger signals and better mass resolution in the negative ion mode as compared to the positive one. This seems to be due to the tendancy to form adducts with Na and K ions giving rise to multiple peaks in the spectrum, even in the presence of diammonium salts. The mass spectra show small signals due to fragment ions that can originate from the loss of one base. The frequency of loss of G, A and C has been observed by using Maldi-MS to characterize oligonucleotide sequences[29].

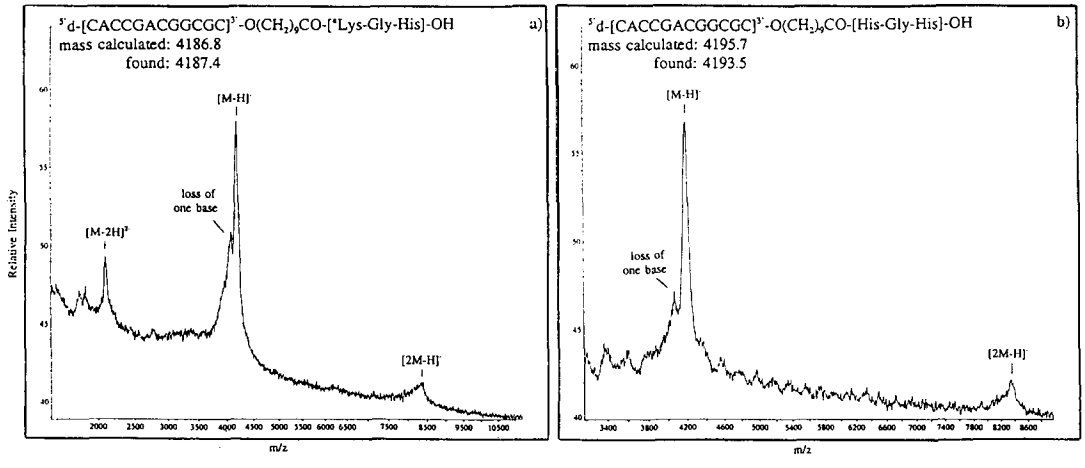

Figure 3 : Molecular weight determinations by negative ion MALDI-TOF MS of the peptide-oligonucleotide conjugates with 2,4,6-trihydroxy acetophenone as matrix and diammonium-L-tartrate. a) conjugate **10a** and b) conjugate **10b**.

CONCLUSION

The data presented here demonstrate that a commercially available Boc-His(Tos)-OH derivative in combination with pentafluorophenyl ester activation is very helpful in the on-line solid phase synthesis of peptide-oligonucleotide conjugates. A mild deprotection with sodium hydroxide followed by reversed-phase chromatography affords the desired products in good yield. The MALDI-TOF MS with 2, 4, 6-trihydroxy acetophenone as the matrix allows the unambiguous characterization of the synthesized conjugates.

EXPERIMENTAL SECTION

Abbreviations Ap = aminopropyl, Boc = t-butyloxycarbonyl, CPG_{500} = Controlled Pore Glass 500, DCC = dicyclohexylcarbodiimide, DCM = dichloromethane, DIEA = diisopropylethylamine, DMAP = dimethylaminopyridine, DMTr = 4,4'-dimethoxytrityl, Dnp = dinitrophenyl, Fmoc = 9-fluorenylmethyloxycarbonyl, HOBt = 1-hydroxybenzotriazole, NMP = N-methylpyrrolidone, Pfp = pentafluorophenyl, Π-Bom = benzyloxymethyl, SPPS = Solid Phase Peptide Synthesis, TEAA = triethylammonium acetate, TFA = trifluoroacetic acid, Tos = tosyl.

Materials Boc protected amino acids and DCC were obtained from Novabiochem and Neosystem. Reagents for peptide synthesis were from Merck except Pfp (SNL). Reagents for oligonucleotide synthesis were purchased from Eurogentec and Milligene. CPG_{500} was from Fluka, 10-hydroxydecanoïc acid, 2,4,6-trihydroxy acetophenone and diammonium-L-tartrate were from Aldrich. All other solvents and reagents were reagent grade and were used without further purification. Analytical TLC was carried out on Merck 5554 Kieselgel 60F 254 plates and eluted with DCM/MeOH (95:5, v/v). Merck 7734 Kieselgel 60 was used for column chromatography. Peptide synthesis were carried out in a manual synthesizer. Oligonucleotide synthesis were carried out on a Pharmacia Gene Assembler via cyanoethyl phosphoramidites chemistry[30]. HPLC was performed with a Waters 600E (System Controller) equiped with a Waters 990 Photodiode Array Detector allowing simultaneous detection at two different wavelengths (220 and 260 nm in our work). Absorption spectra were recorded on an Uvikon 860 (Kontron). ^{1}H NMR spectra were recorded on a Bruker AM 300 with tetramethylsilane as internal standard. Mass spectra were run on a Lasermat time-of-flight commercialized by Finnigan MAT.

10-[di-(*p*-methoxyphenyl) phenyl methyloxy]-decanoic acid

Dimethoxytrityl chloride (6.78 g, 20 mmol) was added to a magnetically stirred solution of 10-hydroxydecanoic acid (1.88 g, 10 mmol) in anhydrous pyridine (30 ml). The reaction was monitored by TLC

analysis using DCM/MeOH (95:5, v/v). After one night at room temperature, the solution was evaporated to dryness. The oily residue was solubilized in ethylacetate and washed with water and saturated sodium chloride. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified on a silica gel column using DCM/MeOH mixture as eluent. 69 % yield as an oil, R*f* = 0.25 DCM/MeOH (95:5, v/v). ^1H NMR ($CDCl_3$) : δ 1.2-1.4 (m; 10H; CH_2), 1.5-1.7 (m; 4H; CH_2), 2.35 (t; J = 7.5 Hz; 2H; H_2), 3.05 (t; J = 6.5 Hz; 2H; H_{10}), 3.7 (s; 3H; OCH_3), 3.8 (s; 3H; OCH_3), 6.75-7.45 (m; 13H; ArH).

Synthesis of pentafluorophenyl esters : General procedure[24]

The N-protected or O-protected derivative (10 mmol) and pentafluorophenol (10 mmol) were dissolved in 75 ml of ethylacetate and the solution was cooled to 0°C. Dicyclohexylcarbodiimide (12 mmol), dissolved in 10 ml of ethylacetate, was added and the reaction mixture was stirred one hour at 0°C under nitrogen. After one night the dicyclohexylurea was filtered and the solvent was removed in vacuo. The resulting oil or solid product was controlled by TLC analysis with DCM/MeOH (95:5, v/v) as eluent (Boc-Gly-OPfp: R*f* = 0.8; Fmoc-Lys(Boc)-OPfp: R*f* = 0.45; $DMTrO(CH_2)_9CO_2Pfp$: R*f* = 0.9; Boc-His(Tos)-OPfp: R*f* = 0.2) and the crude ester was used for coupling reaction without further purification.

Solid phase synthesis

Derivatization of the Controlled Pore Glass support

The preparation of derivatized 3-aminopropyl Controlled Pore Glass 500 **2** was run according to the standard procedure[30]. $DMTrO(CH_2)_9CO_2Pfp$ **1** (240 mg, 0.36 mmol) and triethylamine (0.10 ml, 0.72 mmol) in 2 ml NMP/DCM (1:1, v/v) were added to Ap-CPG_{500} **2** (0.3 g, 30-40 µmol NH_2). The degree of functionalization was quantified by spectrophotometric assay of the dimethoxytrityl cation (λ = 495 nm, ε = 71700 M^{-1}) released after $HClO_4$ treatment of a small aliquot of support. The loading obtained was 38 µmol/g support. Residual unreacted amino groups were acetylated by treating the CPG with acetic anhydride (0.15 ml, 1.6 mmol) in the presence of DMAP (20 mg, 0.16 mmol) in 1.5 ml NMP/DCM (1:1, v/v) for 30 min.

Coupling of the histidine residue

Functionalized CPG was treated with 3 % dichloroacetic acid in 1,2-dichloroethane (2×5 min), washed with 1,2-dichloroethane and with DCM. It was treated with a solution of Boc-His(Tos)-OH (661 mg, 1.5 mmol), DCC (319 mg, 1.55 mmol) and DMAP (18.6 mg, 0.15 mmol) in 6 ml of NMP/DCM (1:1, v/v) for 24 hours. After several washings with NMP, DCM and MeOH, the extent of reaction was determined by spectrophotometry assay of the tosyl group (λ = 221 nm, ε = 14200 M^{-1}) released after NaOH treatment of a small aliquot of the support. A loading of 33 µmol/g support was obtained. Residual unreacted hydroxyl groups were acetylated with Ac_2O as described above.

Coupling reactions via pentafluorophenyl esters

Boc groups were removed from the histidine residues with trifluoroacetic acid (30 % TFA in DCM) for 30 min. After several washings with DCM, a neutralization step with DIEA (5 % in DCM) (2x10min.), and more washings with DCM, the supported peptide was dried under vacuum and stored under nitrogen. Pentafluorophenyl active esters were then coupled as follow : The peptidyl-CPG was swelled with 0.5 ml of NMP/DCM (1:1, v/v) under nitrogen. 40 eq. of freshly prepared crude activated ester in 1.5 ml NMP/DCM were added to the supported peptide followed by 40 eq. of triethylamine freshly distilled on KOH. The mixture was occasionally shaken for 2 hours, after which the supernatant was removed, the support was washed with NMP (3x4 ml), DCM (3x40ml) and residual unreacted amino or hydroxyl groups were acetylated. The extent of the reaction was determined either by spectrophotometric assay of the tosyl group (His) released by NaOH, the Fmoc group (Lys) released by piperidine treatment (λ = 301 nm, ε = 7800 M^{-1}) or of the DMTr cation released by $HClO_4$ from a small amount of the support. Measurement of the DMTr for compounds **8a** and **8b** gave respectively 30 and 29 µmol/g support.

Oligonucleotide synthesis

After synthesis of intermediates **8a** and **8b**, oligonucleotide syntheses were run on a Pharmacia synthesizer using the phosphoramidite method[26].

Deprotection of the conjugates 9a and 9b

The 5' HO-supported peptide-oligonucleotides were treated with 0.1 M sodium hydroxide (1 M NaOH/MeOH/H_2O, 1/6/3, v/v/v). **9a** or **9b** was introduced in a vial and 2 ml of the cleavage solution were added. After 2 hours, (the time required to cleave the ester linkage between the conjugate and the support), the supernatant was recovered and the support was washed twice with 1 ml 0.1 M NaOH. The combined supernatants were left for 24 hours at room temperature (the time required to complete the deprotection of the nucleotide bases). The solution was neutralized at pH 7 by acetic acid and was concentrated under vacuum to give the fully deprotected crude conjugates.

Purification and quantification of conjugates 10a and 10b

The crude conjugates were analyzed by reversed-phase HPLC on a Lichrospher 100 RP 18 (5 µm) column (125x4 mm) eluted with a linear gradient of acetonitrile in 0.1 M aqueous triethylammonium acetate from 0 to 30 % B in 30 min (eluent A = 100 mM TEAA, pH 7, 5 % CH_3CN; eluent B = 100 mM TEAA, pH 7, 80 % CH_3CN), with a flow rate of 1 ml/min. Reversed-phase chromatography was carried out on a 250 mmx10 mm column packed with 10 µm Lichrospher RP 18 from Merck. The elution system employed for purification of the conjugates was the same as the one used for the analytical procedure but the flow rate was raised to 4 ml/min. Products **10a** and **10b** were then quantified by UV spectrophotometry at 260 nm (**10a**, t_R

= 16.7 min., 17.3 O.D., 14.2 % yield; **10b**, t_R = 18.5 min., 30.1 O.D., 18.5 % yield).

Characterization of the conjugates 10a and 10b

Ion-molecular weights of the conjugates were determined by mass spectrometry using a Lasermat time-of-flight instrument (LD-TOF). The average power of the nitrogen laser (337 nm) was about 10^7 W/cm^2. The laser produced 3 ns pulses of approximately 100 µJ in energy. The laser beam is focused down on to a spot on the target appromixately 0.1 mm by 0.3 mm. To improve the signal to noise ratio, 10 to 20 single shot spectra were accumulated and averaged. All measurements were performed using negative detection mode. The spectrometer was calibrated with the $[M-H]^-$ and $[2M-H]^-$ mass peaks of dT_{12} as reference.

Sample preparation. 10 µl of a 0.5 M solution of 2,4,6-trihydroxy acetophenone in ethanol, 5 µl of a 0.1 M aqueous solution of diammonium-L-tartrate were mixed. 1 µl of the conjugate containing solution (10 OD/ml) was added and the mixture was briefly vortexed. 1 µl of this solution was applied to the probe tip and the solvents gently removed in vacuum.

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