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A Method for Solid-Phase Synthesis of Oligonucleotide 5′-Peptide-Conjugates Using Acid-Labile α-Amino Protections

Simone Zaramella,† Esther Yeheskiely, and Roger Strömberg*

Contribution from the Division of Organic and Bioorganic Chemistry, MBB, Scheele Laboratory, Karolinska Institutet, S-17177 Stockholm, Sweden

Received May 24, 2004; E-mail: roger.stromberg@mbb.ki.se

Abstract: We describe the development of a solid-phase technique for the synthesis of 5′-peptide−oligonucleotide conjugates (POCs) with a uniform protection strategy for the nucleic acid and the peptide fragments. On the α-amino function, the amino acid building blocks were protected with the 2-(biphenyl-4-yl)propan-2-yloxycarbonyl (Bpoc) group. This protection is removed during the stepwise peptide elongation by the same acidic conditions used for removal of the dimethoxytrityl (DMT) group used in the oligonucleotide assembly (3% trichloroacetic acid, 2 min). The 2-(3,5-dimethoxyphenyl)propan-2-yloxycarbonyl (Ddz) group was also tested. With this somewhat more stable group, a prolonged contact with the acid (at least 16 min) was required for accomplishing complete α-amino deprotection, which resulted in some degree of depurination of the acid-sensitive DNA chain. Base-labile acyl protections were adopted for the side-chains of histidine, lysine, and the nucleobase amino functions. These were all removed in the final deblocking step by ammonolysis. This uniform protection scheme for the peptide and the oligonucleotide enabled the total stepwise synthesis of model conjugates in the 3′ → *N* direction with high efficiency and purity.

Introduction

Oligonucleotides and their analogues are well-established synthetic tools for the regulation of gene expression in model systems.[1,2] However, their poor cellular uptake, unfavorable intracellular trafficking, and susceptibility to enzymatic degradation reduce their efficacy as antisense agents in vivo. In this context, the covalent attachment of a peptide to an oligonucleotide has been shown to enhance the biological effects of antisense molecules.[3,4] In particular, oligonucleotides tethered with peptides possessing membranotropic activity were internalized by cells with some efficiency as compared to unconjugated ones.[5−11] Moreover, intracellular localization signals linked to oligonucleotides were capable of directing the conjugate toward different cellular compartments.[6,12,13] Another interesting application is peptide−oligonucleotide conjugates (POCs) with a peptide part capable of catalyzing the cleavage of phosphodiester bonds,[14] such hybrid molecules thereby acting as artificial nucleases. In this respect, histidine-containing peptides were considered of particular value because of the catalytic role of imidazole residues in phosphodiester hydrolysis.[15,16]

Over the past years, several research groups have been engaged in developing chemical methods for the preparation of POCs.[3,17] Two different strategies have been described: (1) the post-assembly conjugation approach, by which peptide and oligonucleotide are separately assembled on different solid-supports, deblocked, and after purification linked by means of chemoselective ligation reactions in solution; and (2) the total stepwise synthesis, whereby the two molecules are subsequently synthesized in a sequential manner on the same solid support and finally deprotected. The major challenge when working with the second approach is to devise a synthetic chemistry compatible with both nucleic acid and peptide fragments. Particular attention must be devoted to finding an adequate combination of protecting groups for the variety of functionalities present in the nucleotides and amino acids. The very acid-sensitive nature of nucleic acids, in particular DNA, prevents the use of most of the amino acid side-chain protections commonly employed

† Present address: AstraZeneca R&D, S-15185 Södertälje, Sweden.

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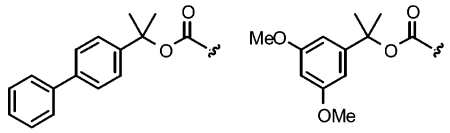
in peptide chemistry, which are typically removed with concentrated strong acids. In contrast, the standard transient α-amino protecting groups, *tert*-butyloxycarbonyl (Boc) and 9-fluorenylmethyloxycarbonyl (Fmoc), can be utilized as long as the peptide is assembled before the oligonucleotide.[15,18−20] This synthetic scheme inevitably results in POCs where the two fragments are connected through the peptide N-terminus and the oligonucleotide 3′-end, unless nucleopeptides[21] are prepared or more complex trifunctional linkers are involved,[22−25] which may result in undesired additional functional groups after deblocking of the conjugate. Whenever a peptide fragment directly tethered to the 5′-end of the oligonucleotide was needed, only the Fmoc-chemistry could be applied.[26] However, the Fmoc-removal conditions are not entirely safe with respect to the oligonucleotidyl-resin, which typically contains base-labile cyanoethyl-protection of the phosphotriester moieties and a succinyl linker between the oligonucleotide and the resin. Hence, to avoid problems, substantial deviations from the standard protocols have been introduced.[27]

In our view, a feasible protecting group strategy for POC synthesis should employ very acid-labile transient protections for both the 5′-hydroxyl and the α-amino functions, whereas the side-chain protections in both peptides and oligonucleotides should be concomitantly removed with the one-step base treatment established for solid-phase oligonucleotide synthesis. Such a protection scheme should enable preparation of conjugates irrespective of the arrangement of the nucleic acid and the peptide fragments, and without the need for complex trifunctional linkers. We have embarked on the development of a protecting group strategy of the aforementioned kind by exploiting as far as possible commercially available materials. Therefore, we considered highly acid-labile α-amino protecting groups already developed for peptide chemistry, such as the 2-(biphenyl-4-yl)propan-2-yloxycarbonyl (Bpoc)[28,29] and the 2-(3,5-dimethoxyphenyl)propan-2-yloxycarbonyl (Ddz),[30] of which several amino acid building blocks are commercially available or can easily be prepared from commercially available reagents.[31]



Bpoc Ddz

In the present report, we evaluate the application of N^{α}-Bpoc- and N^{α}-Ddz-amino acids in solid-phase stepwise synthesis of 5′-peptide−oligonucleotide conjugates. This study includes the optimization of a synthetic protocol based on a simple model POC and its application to the assembly of longer POCs containing trifunctional amino acids.

Results and Discussion

Bpoc and Ddz protecting groups are normally removed with diluted trifluoroacetic acid (TFA) solutions (0.2−3%) in a few minutes. We reasoned that even the 3% trichloroacetic acid (TCA) solution commonly used for cleavage of the dimethoxytrityl (DMT) group in oligonucleotide chemistry could suffice for complete deprotection. The permanent side-chain protection scheme involves base-labile acyl groups for the exocyclic amino groups of the nucleobases (N^4- and N^6-benzoyl for cytosine and adenine, respectively, and N^2-isobutyryl for guanine) and for the side-chains of the two trifunctional amino acids employed in this study (histidine and lysine). As a part of a project aiming at the development of artificial nucleases, we were particularly interested in the synthesis of histidine-containing POCs. Despite the variety of protecting groups available for the imidazole function of histidine, none seemed to completely fulfill the protection strategy requirements. Therefore, we have earlier developed a protection for the histidine side-chain, the 2,6-dimethoxybenzoyl group (2,6-Dmbz), that is compatible with Fmoc, Boc, Bpoc, and DMT chemistries, and that is removed by ammonolysis.[32] The histidine derivative Bpoc-His(2,6-Dmbz)-OH tested in conjugate synthesis was prepared in high yields according to a previously described procedure.[29,32] Because many of the carrier peptides utilized in oligonucleotide delivery into cells contain lysine, its incorporation in POC sequences is clearly relevant. It has been shown that the lysine side-chain can be suitably protected with trifluoracetyl (Tfa) or Fmoc.[33] We prepared the corresponding derivative Bpoc-Lys(Tfa)-OH following established procedures.[29] The protection strategy was completed by utilizing the standard cyanoethyl (CE) group for the internucleoside phosphate moieties of the oligonucleotide.

N-Acylation Side-Reactions During POC Synthesis. First, a set of conditions was chosen as the starting protocol for an exploratory experiment. The oligodeoxynucleotide (ODN) was assembled on a standard preloaded controlled pore glass (CPG) column with a succinyl linker between the resin and the first nucleoside. Conventional cyanoethyl-phosphoramidite chemistry was employed on a DNA synthesizer. To provide a linker-free anchorage for the peptide elongation, 5′-amino-5′-deoxythymidine was incorporated as the last nucleotide.[26] A continuous-flow DNA synthesizer (henceforth referred to as the peptide synthesizer) was reconfigured for running peptide synthesis by the Bpoc-chemistry. Because in some reports the acylation of

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Table 1. POC Sequences and Characterization by ESI Mass Spectrometry

POC	sequence[a]	mass[b] (calcd/found)
Pep1-ODN1	^{N}GlyAlaAlaGly^{C}—5′**T**GCTAGAGAT3′	3322.3/3323.0
Pep2-ODN2	^{N}AlaPheGlyAlaAlaGly^{C}—5′**T**ACCTAATTAGACGGT3′	5353.8/5350.0
Pep3-ODN3	^{N}HisGlyHisGly^{C}—5′**T**GCTAGAGATTTTTAC3′	5273.6/5274.0
Pep4-ODN3	^{N}AlaLeuLysAlaAlaLysLeuAla^{C}—5′**T**GCTAGAGATTTTTAC3′	5652.2/5648.3

[a] Bold T denotes 5′-amino-5′-deoxythymidine. [b] Molecular weights are reconstructed from the multiply charged peaks.

the 5′-aminothymidine residue is reported to be somewhat difficult, we applied a double coupling protocol for the first peptide cycle.[26,27] The removal of Bpoc was carried out by treatment with 3% TCA in dichloromethane (2 × 1 min). Initially, a neutralization step with *N*,*N*-diisopropylethylamine (DIEA) in dichloromethane after acidolysis was included. N^{α}-Bpoc-amino acids were dissolved in the presence of 2 equiv of DIEA to avoid self-catalyzed Bpoc-cleavage and were activated (5 min) prior to coupling. Condensation was mediated by *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), using a coupling time of 1 h.[34] After removal of the last Bpoc group,[35] the POC-resin was finally subjected to ammonolysis with 32% aqueous ammonia at 55 °C for 12 h. The crude product was analyzed by HPLC, and the eluates corresponding to the major peaks were collected and subjected to mass analysis in an electrospray ionization-time-of-flight (ESI-TOF) mass spectrometer.

In the first attempt, chromatographic and mass spectral analysis revealed the crude material to be mostly 5′-acetylated oligonucleotide (entry 1, Table 2). Alongside this, 5′-benzoylated and 5′-isobutyrylated oligonucleotides were also detected. Because peptide elongation was performed without capping, the source of acetyl groups had to be the capping mixture used in the oligonucleotide synthesis, possibly from acetylated nucleobases. This side-reaction has been previously noticed in another approach for the synthesis of 5′-POCs,[36] and in our case this may have been worsened by the neutralization step. A more careful evaluation of the conditions that influence this side-reaction was performed on the model conjugate **Pep1-ODN1** (see Table 1 for the sequence of POCs prepared in the present study). The problem of *N*-acetylation was tackled by (a) replacing the neutralization step with in situ neutralization (addition of 3 equiv of base as compared to resin loading in the coupling mixture),[37] (b) omitting the capping on the last nucleotide cycle, and (c) introducing a brief treatment with 2% morpholine in acetonitrile to consume sufficiently reactive acetyl species before the peptide elongation. Introducing only the first two safety measures together still gave predominantly the acetylated oligonucleotide (39%) (entry 2, Table 2), whereas the simultaneous introduction of all of these precautions led to a substantial reduction of the side-product to 11−12% of the total crude (entries 3 and 4, Table 2). An attempt to further reduce this value by washing with a stronger nucleophile, such as piperidine, led only to a marginal improvement (<9%) (entry 5, Table 2). From the analysis of the crude ODN, it was found that a certain amount of side-product (accounting for 8−9% of the total crude) was already present before starting the peptide assembly, and therefore it could not be ascribed to conditions found during peptide synthesis cycles. Despite its steric hindrance, it appeared that the monomethoxytrityl (MMT) protection only partially prevents the reactivity of the 5′-amino group toward the acetylated nucleobases. Therefore, we introduced the nucleophilic washing step already at the oligonucleotide cycle prior to the last one. Whereas the treatment with piperidine in this fashion led to the almost complete disappearance of the acetylated ODN (1%) and to the appearance of an unidentified side-product (∼6%) (entry 7, Table 2), the synthesis with the morpholine wash was devoid of this new side-product as well as of the acetylated ODN (<1%) (entry 6, Table 2). Alternatively, complete removal of the capping step throughout the oligonucleotide synthesis[36] did not give any detectable N-acetylation (entry 8, Table 2).

Table 2. Extent of N-Acetylation of 5′-Amino-ODN during Peptide−Oligonucleotide Conjugate Synthesis

entry	method	5′-Ac-ODN [%][a]
1	after standard ODN synthesis[b]	60 (11)
2	no capping on last ODN cycle[b]	39 (23)
3	no capping on last ODN cycle + morpholine wash (1 min)[b]	11 (41)
4	no capping on last ODN cycle + morpholine wash (2 min)[b]	12 (45)
5	no capping on last ODN cycle + piperidine wash (1 min)[b]	8.6 (54)
6	no capping on last ODN cycle + morpholine wash at $n-1$ ODN cycle[c]	0.8 (61)
7	no capping on last ODN cycle + piperidine wash at $n-1$ ODN cycle[b]	1.0 (56)[d]
8	no capping during ODN synthesis[c]	n.d. (62)

[a] From the integration of the HPLC peak areas measured at a UV wavelength of 260 nm. Numbers in brackets are the amounts of the target POC **Pep1-ODN1**. [b] POC synthesis at 1-μmol scale from an aliquot of ODN-resin prepared at 10-μmol scale. [c] POC synthesis at 1-μmol scale from ODN-resin prepared at 1-μmol scale. [d] A considerable amount of an unidentified side-product was detected (5.7%).

The syntheses without nucleophilic washes or with the post-ODN assembly washes yielded an amount of 5′-Bz- and 5′-*i*Bu-ODN varying between 8% and 12%. With the washes at the $n-1$ ODN cycle, the amount of acylated side-products was reduced to 6−7%. In the absence of any capping step, the side-products accounted for 8% of the crude product, indicating that the N-acylation was little related to the capping procedure but it, although surprising, possibly originates from direct reaction of the 5′-amino function with the nucleobase protecting groups. Such behavior has been observed for phenoxyacetyl

(34) The design of the continuous-flow peptide synthesizer comprises a rather large coupling loop (0.5 mL) that includes the pump and the column; therefore, to achieve a functional coupling concentration (0.04 M) with the 1-μmol scale, a large excess of activated monomers had to be used (20 equiv).
(35) Initially, the Bpoc was retained after the final coupling cycle to provide a purification handle for the POC in analogy with the "DMT-on" purification protocol in oligonucleotide chemistry. However, the Bpoc-removal required a prolonged treatment with 80% HOAc (3−4 h), which we feared would cause extensive depurination. Therefore, this approach was later skipped and Bpoc-removal was performed prior to ammonolysis by a standard TCA treatment.
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Table 3. Yields of Peptide−Oligonucleotide Conjugate Syntheses with Different Peptide Synthesis Protocols

entry	peptide chemistry[a]	method[a]	solid support[b]	yield [%][c]
1	Bpoc-AA/HBTU/DIEA	standard cycle	CPG #1	50
2	Bpoc-AA/HBTU/DIEA	3 × 1 min TCA	CPG #1	49
3	Bpoc-AA/**HATU**/DIEA	standard cycle	CPG #1	51
4	Bpoc-AA/**HATU**/DIEA	coupling 30 min	CPG #1	54
5	Bpoc-AA/HBTU/**NMM**	standard cycle	CPG #1	57
6	Bpoc-AA/HBTU/**TMP**	standard cycle	CPG #1	54
7	Bpoc-AA/HBTU/**NMM**	coupling 30 min	CPG #1	55
8	Bpoc-AA/HBTU/**NMM**	resident reagents	CPG #1	52
9	**Ddz-AA**/HBTU/**NMM**	2 × 8 min TCA	CPG #2	46
10	**Bpoc-AA-OPfp**/DIEA	double coupling 2 h	CPG #2	42
11	Bpoc-AA/HBTU/**NMM**	resident reagents	CPG #2	55
12	Bpoc-AA/HBTU/**NMM**+**DBU**	resident reagents	CPG #2	59
13	Bpoc-AA/HBTU/DIEA	standard cycle	**HCL PS**[d]	68

[a] Bold letters identify the variations in the starting chemistry protocol taken as reference, as defined in entry 1. Under the method column, only the modification introduced in the standard cycle is reported. The standard cycle is 2 × 1 min 3% TCA, 60 min coupling (preactivated amino acids). [b] #1 defines a batch of ODN-resin prepared at 10-μmol scale without capping during ODN synthesis; #2 defines a batch of ODN-resin prepared at 10-μmol scale with the method described in entry 7 of Table 2. [c] Amount of the target **Pep1-ODN1** from the integration of the HPLC peak areas measured at a UV wavelength of 260 nm. [d] High cross-linked polystyrene resin; synthesis performed at 1-μmol scale.

protection of nucleobases[26] but has not been reported for Bz and *i*Bu protecting groups.

Optimization of the Synthesis of the Model Conjugate with N^{α}-Bpoc-Amino Acids. After having eliminated the problem of the N-acetylation of the 5′-amino group, an investigation of factors (i.e., coupling reagent, base, coupling time, and solid-support) that can affect the POC synthesis was performed. To test various peptide synthesis conditions, aliquots corresponding to 1 μmol of the oligonucleotidyl-resin prepared in a 10-μmol scale[38] were transferred to a suitable column and installed on the peptide synthesizer (refer to the Experimental Section for a detailed description of the peptide assembly). In accordance with what has been reported by others,[26] a fraction of 5′-amino-ODN-resin remained unreacted after the coupling of the first amino acid (in our experiments, up to 2−3%), even with extended coupling times (entry 5 vs entry 7, Table 3). Use of the more reactive coupling reagent *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) instead of HBTU did not give any appreciable improvement (entry 1 vs entry 3, Table 3). Notably, HATU gave a slightly better synthesis with the coupling time reduced to 30 min (entry 4, Table 3), perhaps because less of the side-reactions associated with the use of HATU take place (e.g., guanidination).

As an alternative to DIEA (pK_a 10.1 and 18.1 in water[39] and in acetonitrile,[40] respectively), weaker bases, such as 2,4,6-trimethylpyridine (TMP) and 1-methylmorpholine (NMM), were employed to minimize the risk of cyanoethyl removal from the phosphate triester moieties of the ODN. The use of NMM and in particular of TMP in peptide synthesis was also reported to be beneficial in minimizing racemization of the activated amino acid.[41] NMM and TMP are bases of similar strength according to the pK_a values in water (7.38 and 7.43, respectively); however, in a polar aprotic solvent, such as acetonitrile, there appears to be a difference of about one pK_a unit (15.7 and 14.8, respectively).[42,43] In our hands, the use of NMM proved to be the best choice (entry 1 vs entries 5 and 6, Table 3). These results may reflect the reduction of side-reactions associated with the use of a strong base. On the other hand, due to the sensitivity of the Bpoc group to acids, the presence of a relatively stronger conjugate acid of the weak base may be detrimental for the integrity of the N^{α}-Bpoc-amino acids. Hence, NMM, being of intermediate strength between DIEA and TMP, may represent the optimal compromise. The influence of the strength of the conjugate acid of the base was indirectly confirmed by an experiment where the predissolved amino acids were resident on the synthesizer in the presence of NMM throughout the synthesis, and not activated and installed prior to the individual coupling. In this case, the yield was slightly lower (entry 11, Table 3). When 1 equiv of NMM was replaced by a much stronger base (hence, a much weaker conjugate acid), such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), a higher yield was obtained (entry 12, Table 3). However, this comparison should take into account the possible difference in the rates of amino acid activation and coupling when using bases of different strengths. It should be noted that the difference between entries 8 and 11 (Table 3), which describe the same experiments but on different ODN-resin batches, also account for the different qualities of the ODNs prepared on the two different resin batches.

The influence of the solid-support was briefly investigated by preparing the model POC on a highly cross-linked polystyrene resin. The 1-μmol-scale synthesis provided a better yield than the CPG-based method (compare entry 6 in Table 2 with entry 13 in Table 3). However, this finding may not be general because it has been shown that the influence of the solid support is much dependent on the sequences of the fragments constituting the POC and perhaps also on the order and the way they are connected to one another.[18−20,33]

To circumvent the problem of the stability in solution as free acids of the Bpoc-building blocks,[44] we considered the use of

(38) A lower quality of the POCs prepared from the oligonucleotidyl-resins synthesized in a 10-μmol scale was generally observed, which accounts for the higher efficiency of the oligonucleotide synthesis performed at 1-μmol scale (average coupling yield >99%, according to the trityl cation release) as compared to that at 10-μmol scale (average coupling yield >94%) (compare entry 6 in Table 1 to entry 1 in Table 2).

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active esters of the N^{α}-Bpoc-amino acids, in particular the pentafluorophenyl (Pfp) esters. The preparation and use of these compounds in peptide synthesis has been thoroughly described, and some advantages are apparent. First, the compounds (mostly crystalline) have higher stability during prolonged storage and in solution.[44] Second, because of the absence of a real mixing chamber in our continuous-flow synthesizer, the activation of the monomers might not be optimal, a potential problem that is overcome by the use of preformed active esters. Unfortunately, Pfp esters of Fmoc-amino acids have been described in POC synthesis as giving somewhat lower coupling efficiencies,[26] and therefore we adopted protocols with extended coupling times (up to 2 h). Nevertheless, yields remained clearly inferior to those with HBTU-mediated condensation even when a double coupling cycle of 2 h was used for each elongation step (entry 10, Table 3).

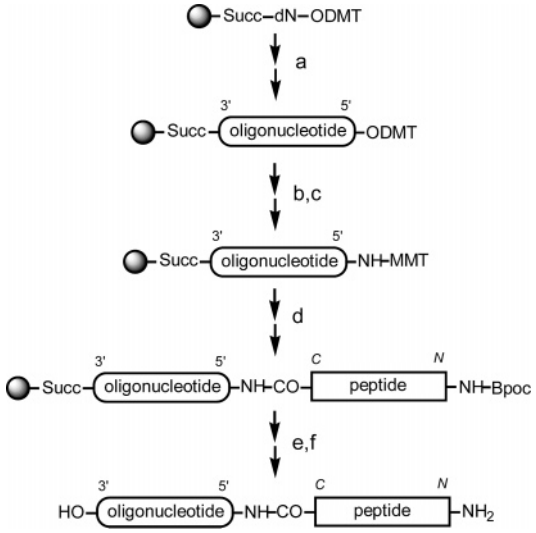
Synthesis of the Model Conjugate with N^{α}-Ddz-Amino Acids. Because of a larger availability of commercial building blocks, the use of N^{α}-Ddz-amino acids was considered to expand the applicability of our POC synthesis strategy. This group is somewhat more resistant to acid than Bpoc, being split with as high as 3% TFA. Nevertheless, we hoped that the flow-through acidic treatment with 3% TCA would be strong enough to promote also the removal of Ddz. Unfortunately, deprotection was not complete even when the acidic treatment was prolonged to 8 min, as a considerable amount of Ddz-protected and unprotected shorter scrambled oligomers was found in the crude product. Encouraged by the suggestion that 15% DCA can effect detritylation faster and with less depurination than with 3% TCA,[45] we applied this mixture for the cleavage of Ddz. In our hands, this treatment provided only a marginal improvement of the deprotection efficiency, at the expense of what appeared to be a large extent of depurination. Only when an acid treatment of 16 min with 3% TCA was applied was complete Ddz-removal attained. As expected, the level of depurination in this case was unacceptably higher than what was normally found with the standard TCA treatment. Despite these results, Ddz-protected amino acids may still be used in the preparation of conjugates of more acid-resistant nucleic acids and their analogues, such as oligoribonucleotides and 2′-*O*-Me-oligoribonucleotides.

Synthesis of More Complex Conjugates. The optimized synthetic protocol described in Scheme 1 and in Table 4 was first tested in the preparation of a longer version of the model POC; the sequence was extended at both sides to incorporate a hexadecamer ODN and a hexapeptide (**Pep2-ODN2**). As a comparison, the same POC was assembled by employing standard Fmoc chemistry for the peptide part.[46] Whereas the synthesis by the Bpoc-based protocol proceeded efficiently with more than 60% of the crude product corresponding to the target conjugate (Figure 1a), it appeared that the Fmoc-based approach led to the formation of only a minor percentage of the target POC (less than 16% of the crude product), giving instead shorter fragments as the predominant products (see Figure S2 in the Supporting Information).

Two more POC sequences were synthesized, one incorporating the His-Gly-His motif (**Pep3-ODN3**),[47] which is known to exert phosphodiester cleavage activity, and one with a peptide capable of permeating cellular membranes (**Pep4-ODN3**).[48] The **ODN3** sequence was complementary to a tract of the Primer Binding Site (PBS) region of genomic RNA of HIV-1. The oligonucleotides were assembled on the DNA synthesizer at 1-μmol scale according to the procedure described in entry 6 of Table 2 (i.e., inclusive of the morpholine treatment before the last nucleotide cycle), followed by the peptide elongation on the peptide synthesizer. The POCs were deprotected and analyzed by ion-exchange and reversed-phase HPLC as well as by mass spectroscopy (Figure 1).

The applicability of the novel protecting group of the histidine side-chain was evaluated in the synthesis of **Pep3-ODN3**. The

Scheme 1. Solid-Phase Synthesis Scheme for the Preparation of 5′-Peptide–Oligonucleotide Conjugates[a]



[a] Reagents and conditions: (a) ODN synthesis, 3% TCA, DCM; DMT-dN CE-phosphoramidite, 1*H*-tetrazole, MeCN; Ac_2O, pyridine, *N*-methylimidazole, THF; I_2, H_2O, pyridine, THF. Last nucleotide cycle: (b) 2% morpholine, MeCN; (c) 3% TCA, DCM; MMT-amino-dT CE-phosphoramidite, 1*H*-tetrazole, MeCN; I_2, H_2O, pyridine. (d) Peptide synthesis: 3% TCA, DCM; Bpoc-AA-OH, HBTU, NMM, DMF. Deblocking and deprotection: (e) 3% TCA, DCM; (f) 32% aqueous ammonia, 55 °C, 12 h.

Table 4. Detailed Conditions for the Solid-Phase Synthesis of the Peptide Part of the POC after the Standard Oligonucleotide Assembly

operations and reagents	time and flow rate
Deprotection	
3% w/v TCA in DCM	2 × 1 min, 2 mL/min[a]
Washing	
DCM	2 min, 2 mL/min
MeCN	2 min, 2 mL/min
DMF	2 min, 1.25 mL/min
Coupling	
Bpoc-AA-OH/HBTU/NMM in DMF (20:19:43, mol/mol/mol)	60 min, recycling 1.25 mL/min
Washing	
DMF	2 min, 1.25 mL/min
MeCN	2 min, 1.25 mL/min
DCM	2 min, 1.25 mL/min

[a] An intermediate 1-min wash with DCM was performed between the two 1-min treatments with TCA.

(45) Septak, M. *Nucleic Acids Res.* **1996**, *24*, 3053–3058.
(46) For this comparison, the oligonucleotidyl-resins of two separate 1-μmol scale ODN syntheses were mixed and then divided into two lots, each corresponding to a loading of 1 μmol. The Fmoc-protocol was entirely similar to the Bpoc-protocol except for the deprotection step, which was performed with a 7-min treatment of 22% piperidine in DMF.
(47) Tung, C.-H.; Wei, Z.; Leibowitz, M. J.; Stein, S. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 7114–7118.
(48) Oehlke, J.; Scheller, A.; Wiesner, B.; Krause, E.; Beyermann, M.; Klauschenz, E.; Melzig, M.; Bienert, M. *Biochim. Biophys. Acta – Biomembranes* **1998**, *1414*, 127–139.

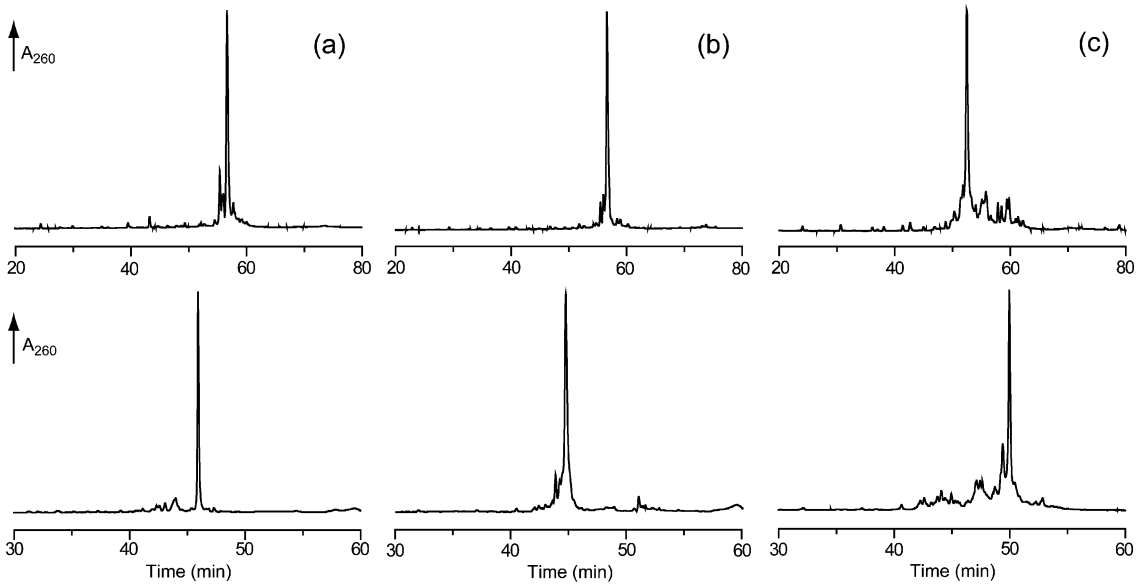


Figure 1. HPLC traces of crude POCs products after deprotection (upper panel ion-exchange, lower panel reversed-phase). (a) **Pep2-ODN2**; (b) **Pep3-ODN3**; (c) **Pep4-ODN3**. IE HPLC was performed on a Dionex Nucleopac PA-100 column (4 × 250 mm) with elution by a linear gradient (5−45%, 78 min) of 0.375 M $LiClO_4$ in 25 mM Tris-Cl (pH 8.3)−30% acetonitrile, with a flow rate of 1 mL/min, a temperature of 50 °C, and UV monitoring at 260 nm. RP HPLC was carried out on a Jones Genesis AQ C18 column (3 × 150 mm) with elution by a linear gradient (0−12%, 46 min) of acetonitrile in 50 mM triethylammonium acetate (pH 6.5), with a flow rate of 0.43 mL/min. The temperature was set at 50 °C, and UV monitoring was performed at 260 nm.

crude product was found to contain 70% of the target molecule according to ion-exchange HPLC analysis (Figure 1b). Various side-reactions are associated with unprotected histidines in peptide assembly, and the use of unprotected monomers is strongly discouraged.[49] Grandas and co-workers reported a comparison between tosyl (Tos), 2,6-dinitrophenyl (Dnp), and unprotected imidazole in the synthesis of histidine-containing nucleopeptides and found that the latter was the best choice.[50] In their work, the synthesis of the peptide preceded that of the nucleic acid fragment and an extra deprotection step to remove the imidazole protection before ODN assembly was needed. In that report, the chromatograms of the crude products showed a much more complex pattern when the nucleopeptides were prepared with N^{im}-Tos- and N^{im}-Dnp-protected histidines, which indicates that these protecting groups may be involved in side-reactions during the conjugate assembly. Although Tos and Dnp have been used in solid-phase POC synthesis by others,[15] their compatibility with various peptide and oligonucleotide synthesis conditions is questionable. Azhayev et al. used in the preparation of histidine-containing POCs a deprotection/reprotection protocol to replace the acid-labile trityl group on the imidazole residue with the base-labile acetyl after peptide elongation and before oligonucleotide synthesis.[51] Our results indicate that the acyl protection here presented can fulfill all of the requirements for a suitable permanent protecting group, that is, stability throughout the entire conjugate assembly, protection against racemization, efficient coupling of the monomer, and ease of removal in the final deprotection.

The peptide incorporated in **Pep4-ODN3** is presumed to adopt an α-helical structure that can promote the cellular membrane permeation by the oligonucleotide. The assembly of **Pep4-ODN3** appeared to have proceeded less efficiently than the previous syntheses (target conjugate 40% of the crude product according to the ion-exchange chromatogram). Although the complex HPLC pattern (Figure 1c) indicates that the coupling was seemingly less efficient throughout the whole synthesis, the mass analysis of the crude conjugate revealed the presence of mainly three products, the target POC being the major entity and two more components having the mass corresponding to the target POC plus one and two lysines, respectively. Furthermore, oligonucleotide degradation may have taken place during the ammonolytic deprotection as previously reported for lysine-containing conjugates.[26]

Conclusion

We have developed an on-line solid-phase synthesis of 5′-peptide−oligonucleotide conjugates on the basis of a common protecting group strategy for both fragments. Acid labile urethanes and trityl-based groups were adopted as transient protections for the α-amino group and the 5′-hydroxyl, respectively. Permanent base-labile side-chain protections were employed for histidine, lysine, and nucleobase amino functions. This methodology enables the solid-phase synthesis of conjugates in the 3′ → *N* direction with high yields and purity. Moreover, the present synthesis scheme provides a possibility to prepare POCs irrespective of the order of the fragments and without changing the set of monomeric derivatives (i.e., the protecting group strategy). In principle, more complex conjugates can be assembled without major changes in the synthesis protocols, for example, peptide−oligonucleotide−peptide and oligonucleotide−peptide−oligonucleotide hybrids. These features can be beneficial for the preparation of POCs libraries by combinatorial solid-phase techniques.

Experimental Section

Bpoc-His(2,6-Dmbz)-OH. Bpoc-His-OH (2.5 mmol, 985 mg), previously prepared according to published procedures,[29] was suspended in anhydrous DMF (5 mL), and DIEA (5.3 mmol, 0.9 mL) was added.

(49) Geiger, R.; König, W. In *The Peptides: Analysis, Synthesis, Biology*; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1981; Vol. 3, pp 70−80.
(50) Beltrán, M.; Pedroso, E.; Grandas, A. *Tetrahedron Lett.* **1998**, *39*, 4115−4118.
(51) Antopolsky, M.; Azhayeva, E.; Tengvall, U.; Azhayev, A. *Tetrahedron Lett.* **2002**, *43*, 527−530.

The slurry was cooled on an ice-bath while a solution of 2,6-Dmbz-Cl (2.6 mmol, 525 mg) in DMF (2 mL) was added dropwise over 15 min. The mixture was stirred for 2 h at room temperature. The bulk of the solvent was evaporated under reduced pressure, and the resulting yellow paste was dissolved in EtOAc (30 mL). The organic phase was washed with 0.5 M citrate buffer (pH 3.5) (30 mL), water (2 × 30 mL), and brine (30 mL). After being dried over Na_2SO_4 and filtered, the organic phase was evaporated to dryness, yielding a white powder, which was triturated with EtOAc/*n*-hexane and then recrystallized from MeCN/Et_2O. White solid (988 mg, 71%). TLC ($CHCl_3$/MeOH/HOAc, 90:8:2, v/v/v): $R_f = 0.38$. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.67 (s, 3H, CH_3), 1.68 (s, 3H, CH_3), 2.79−2.97 (m, 2H, C_β−H), 3.73 (s, 6H, OCH_3), 4.12−4.17 (m, 1H, C_α−H), 6.84 (d, $J = 8.5$ Hz, 2H, Dmbz), 7.31 (br, 1H, im), 7.34−7.39 (m, 3H, NH + Bpoc), 7.44−7.48 (m, 3H, Bpoc), 7.51−7.57 (m, 3H, Bpoc + Dmbz), 7.64 (d, $J = 7.6$ Hz, 2H, Bpoc), 7.88 (br, 1H, im) ppm. ^{13}C NMR (100 MHz, DMSO-d_6): δ 29.9, 30.0, 30.3, 53.8, 56.9, 80.6, 105.4, 111.8, 114.5, 125.6, 127.2, 127.4, 128.2, 129.8, 133.9, 138.2, 139.1, 140.8, 141.2, 146.9, 155.6, 157.9, 163.6, 174.2 ppm. HRMS (ESI-TOF): *m*/*z* calcd for $C_{31}H_{32}N_3O_7$ $[M + H]^+$ 558.6111; found 558.3910.

Oligonucleotide Synthesis. Oligonucleotide synthesis was carried out on an Applied Biosystems A392 synthesizer. ODNs were assembled on preloaded CPG cartridges with standard CE-phosphoramidite chemistry [5′-DMT-dN-3′-P(OCE)N*i*Pr$_2$, dN = T, A^{Bz}, C^{Bz}, G^{iBu}] up to the $n - 1$ cycle, where n is the number of nucleotides for a given ODN. The column was then transferred on the continuous-flow peptide synthesizer and treated with 2% morpholine in acetonitrile (1 min, 1.25 mL/min). After extensive washing with acetonitrile, the column was placed back on the DNA synthesizer for the last elongation cycle with the 5′-MMT-amino-5′-deoxythymidine CE-phosphoramidite, where the capping step was omitted. Alternatively, the DNA assembly was conducted without the capping steps throughout the synthesis. The MMT group was kept on the oligonucleotidyl-resin (ODN-resins were stored cold and dry) and removed by acidolysis as the first step of peptide assembly. For the model POC tests, aliquots of the oligonucleotidyl-resin corresponding to a loading of 1 μmol were transferred on a Pharmacia column for the peptide synthesis. For the longer POCs, the original 1-μmol cartridges were mounted directly on the peptide synthesizer by means of proper adapters.

Peptide Synthesis. Peptide assembly was performed on a Pharmacia Gene Assembler in-house reconfigured for peptide synthesis in continuous-flow mode. Removal of the transient α-amino protecting groups was performed with 3% TCA/DCM (2 × 1 min). The acidolysis was performed at nearly the maximum flow rate allowed by the synthesizer (2 mL/min), in the absence of any scavenger. This was followed by extensive washing with solvents of different polarity. Coupling was performed with amino acid/condensing reagent/base (20:19:43, mol/mol/mol) in DMF (1 h), with an amino acid final concentration of 0.04 M. For the model POC syntheses, amino acids derivatives were preactivated (5 min) before the coupling. At this stage, the procedure was as follows: the synthesis was interrupted during a safe step (washing with DMF) that closely precedes the coupling step; the coupling mixture described above was carefully primed in the synthesizer, and the synthesis was resumed. For the longer POCs, amino acids were installed predissolved in DMF in the presence of the base (except for Bpoc-Gly-OH that was activated prior to coupling because of its poor solubility in DMF either as free acid or in its salt forms). Upon coupling, the condensing reagent and the solution of the monomer with the base are delivered in segments from the different reservoirs and pushed onto the column; the valves then close a recycling loop that is comprised of the pump, short tubings, and the column. The coupling mixture then circulates into this loop for the given reaction time. Extensive washing followed the coupling step. After the final Bpoc-cleavage, the resin was removed from the column, dried under reduced pressure, and incubated in 32% aqueous ammonia (2 mL/30 mg resin) for 12 h at 55 °C in a tightly sealed jar. After being cooled, the slurry was filtered on a glass-sintered funnel, diluted with water, and the excess ammonia was evaporated under reduced pressure. The aqueous solution was freeze-dried, and the crude residue was dissolved in deionized water for chromatography and mass analysis.

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Supporting Information Available: General methods of the Experimental Section; HPLC traces concerning the synthesis optimization of **Pep1-ODN1**; and comparison of the HPLC traces of **Pep2-ODN2** prepared by Bpoc- and Fmoc-chemistry. This material is available free of charge via the Internet at http://pubs.acs.org.

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