Photocleavage of Peptides and Oligodeoxynucleotides Carrying 2-Nitrobenzyl Groups

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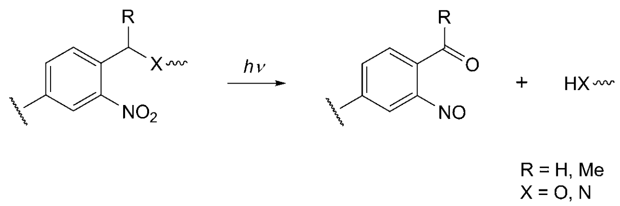
Peptides and oligodeoxynucleotides containing photolabile 2-nitrobenzyl groups as mid-sequences were prepared. Photocleavage of aqueous solutions of these compounds neared completion within 30 min to a few hours depending on the photolabile group used. A photolabile group was introduced in the loop of an intramolecular oligodeoxynucleotide hairpin. Melting curves of the hairpin with and without the complementary oligodeoxynucleotide showed a preference for the intramolecular hairpin form, but an intermolecular duplex was observed after photolysis. These results open the possibility of using photolabile DNA hairpins for the fabrication of patterned surfaces.

Introduction. – Photolysis has been used extensively in organic chemistry and biochemistry. Several molecular groups are light-sensitive, and this property has been exploited to mask groups that are to be liberated at a particular time. 2-Nitrobenzyl groups are some of the most widely studied photolabile groups [1–2]. In the photolysis of 2-nitrobenzyl derivatives, the NO_2 group is reduced to NO, and the benzyl CH_2 group is oxidized to C=O (*Scheme 1*) [3–4]. Several derivatives of the 2-nitrobenzyl group have been used for the protection of amines, alcohols, carboxylic acids, imidazoles, phenols, and phosphates [2][5]. In some cases, low yields attributable to the reaction of the nitroso aldehyde with the amino groups have been reported [5]. The introduction of a Me group in the benzylic position accelerates photolysis and minimizes secondary reactions due to the formation of a methyl ketone that is less reactive than the aldehyde group [2][5]. The 2-nitrobenzyl group has also been used as a photolabile link between peptide and the solid support in solid-phase synthesis [6–8]. The photolytic cleavage of the peptide supports allows the isolation of protected

Scheme 1. *Synthesis of a Photolabile Linker Used for the Introduction of 2-Nitrobenzyl Groups in Peptides*

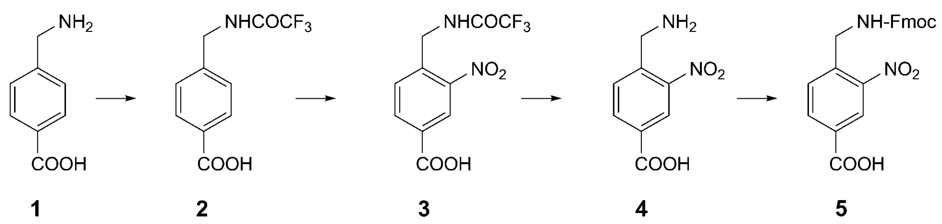


peptide segments for use in the synthesis of longer peptides [7][9]. Moreover, photolabile groups are also used to analyze drug–protein interactions. In this case, masked or caged drug derivatives are synthesized and delivered to cells. Fast photoremoval of the masking groups allows the study of the *in vivo* behavior of the drug [10]. Finally, photolabile groups are used for the protection of the 5′-end of nucleoside derivatives in the preparation of DNA chips. This technique allows the parallel synthesis in a single chip of thousands of 20–30mer fragments corresponding to a complete chromosome [11].

Here, we report the synthesis and photolysis of synthetic biomolecules (peptides and oligodeoxynucleotides) carrying a photolabile link in the middle of their sequence. The ultimate aim of this work is to link these photolabile molecules onto surfaces to obtain functionalized surfaces. Photolithography of these surfaces will result in the cleavage of the biomolecules in the illuminated area, while, in the masked area, the biomolecules will remain intact. Utilizing the self-assembling properties of these photolabile biomolecules will enable selective binding of various molecules to the cleaved or uncleaved biomolecules in the photolyzed and unphotolyzed areas, respectively.

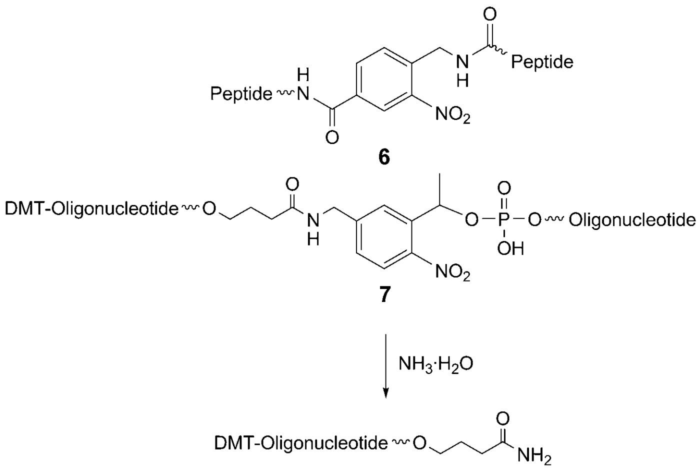
Results and Discussion. – 1. *Synthesis of Photolabile Linkers.* First, an amino acid derivative carrying 2-nitrobenzyl was prepared (*Scheme 2*). We selected a derivative of 4-(aminomethyl)-2-nitrobenzoic acid described in [8][12][13]. The synthesis of this compound was achieved according to the literature [12][13] with small changes. First, the NH_2 group of 4-(aminomethyl)benzoic acid (**1**) was protected by the acid-stable trifluoroacetyl (TFA) group using ethyl trifluoroacetate (*Scheme 2*). Nitration of the resulting compound led to excellent yields of the TFA derivative, **3**, of 4-(aminomethyl)-2-nitrobenzoic acid, which was subsequently hydrolyzed with NH_3 to give compound **4**. Finally, the (9*H*-fluorenyl-9-methoxycarbonyl (Fmoc) derivative, **5**, of 4-(aminomethyl)-2-nitrobenzoic acid was prepared. This compound is suitable for the incorporation of 2-nitrobenzyl groups in peptides using solid-phase peptide protocols.

Scheme 2. *Synthesis of the Photolabile Linker Needed for the Incorporation of 2-Nitrobenzyl Linkers in Peptides*



2. *Synthesis and Photolysis of Peptides Carrying Photolabile Linkers.* The peptide sequence TyrAla-**6**-PheLysGly (*Scheme 3*) was prepared. Standard Fmoc chemistry and solid-phase protocols were applied. The desired peptides were purified by reversed-phase (RP) HPLC and characterized by mass spectrometry.

Scheme 3. *Structure of Photolabile Linkers* **6** *and* **7** *and Hydrolysis Product Found During the Purification of the DMT-Oligonucleotide Hairpin*



Aqueous solutions of the peptide sequence were irradiated with a *Black Eye* lamp (340 nm). The progress of the reaction was followed by HPLC/MS analysis of aliquots taken at different times. Photolysis of the peptide was nearly complete after 2 h of irradiation (*Fig. 1*). The N-terminal fragment without the 2-nitrosobenzaldehyde group was identified at the beginning of the chromatogram, but the tripeptide carrying the 2-nitrosobenzaldehyde group was not observed. Instead, several small peaks appeared along the chromatogram. It has been reported that the 2-nitrosobenzaldehyde group may further react with amino groups [5][14]. This side reaction prevents the isolation of the moiety carrying the 2-nitrosobenzaldehyde group.

3. *Synthesis and Photolysis of a Model Oligodeoxynucleotide Carrying a Photolabile Linker.* Next, an oligodeoxynucleotide carrying a photolabile group mid-sequence was prepared. Oligodeoxynucleotide sequence A, 5′-d(CCCCC-**6**-TTTT), was prepared on a 1-μmol scale. The phosphoramidite **8** shown in *Fig. 2* was used to introduce the photolabile 2-nitrobenzyl group at the midpoint of the sequence. This phosphoramidite has a Me group at the benzyl position, which increases the reaction rate and prevents side reactions, as the methyl ketone group resulting from photolysis is less reactive than the aldehyde group. This compound **8** was obtained from commercial sources. After ammonia deprotection, the resulting oligodeoxynucleotide was purified by RP-HPLC. Oligodeoxynucleotide A eluted at 10.2 min (*Fig. 3*) as a broad double peak. The double peak was attributed to the presence of two diastereoisomers on the phosphoramidite used. The purified compound was characterized by mass spectrometry (MALDI). In addition to the expected mass, we observed two peaks corresponding to the fragmentation of the oligodeoxynucleotide (T_4 and C_5-photolabile) that occurred during the acquisition of the spectra.

Oligodeoxynucleotide A was photolyzed with a *Black Eye* lamp (340 nm) for 30 min at room temperature. *Fig. 3* shows the HPLC profile of samples taken at the start of the photolysis and after 30 min of photolysis. Photolysis was almost completed

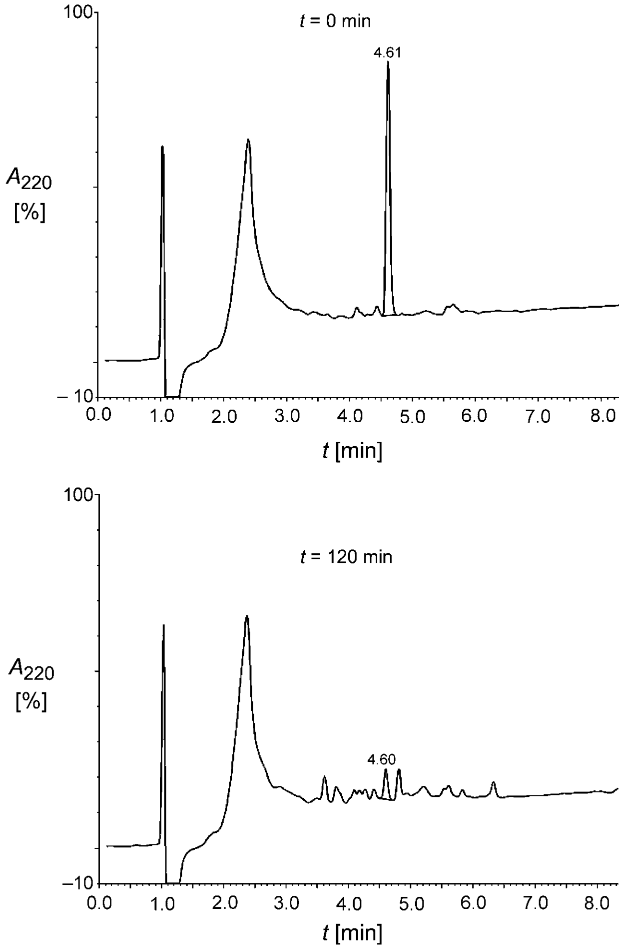


Fig. 1. *Photolysis of peptide H-^{N}Tyr-Ala-***6***-Phe-Lys-Gly^{C}-OH.* HPLC Profiles of samples at the start (t = 0) and after 120 min of photolysis.

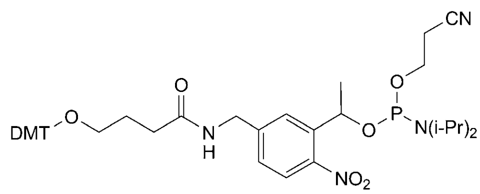


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Fig. 2. *Phosphoramidite derivative* **8** *used for the incorporation of 2-nitrobenzyl linkers in oligonucleotides*

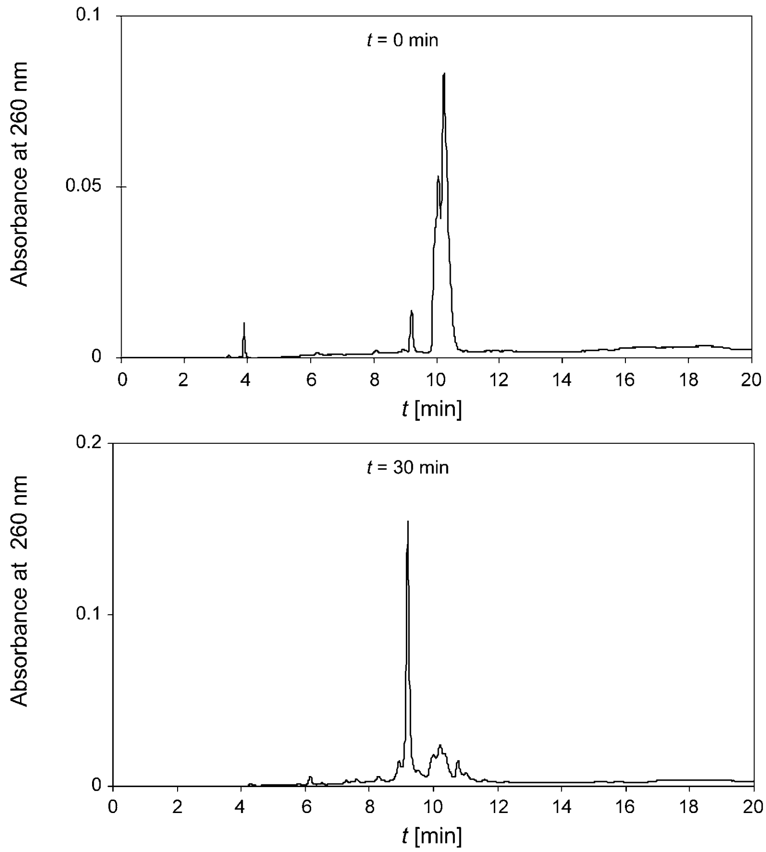

Fig. 3. *Photolysis of oligonucleotide 5′-CCCCC-***7***-TTTT-3′* (Sequence A). HPLC Profiles of samples at the start ($t = 0$) and after 30 min of photolysis.

after 30 min, as judged by the disappearance of the starting oligodeoxynucleotide. As the peak corresponding to sequence A disappeared, a new peak at 9 min was observed, which was identified as T_4 by HPLC analysis of the enzyme digestion, giving thymidine as a single compound. The product containing five cytosines and the 2-nitrosophenyl ketone group was not isolated. We speculate that the photolabile C_5-moiety undergoes further reactions, yielding several compounds. The presence of the Me group at the benzyl position accelerated the photolysis, but it did not prevent the formation of side-products in the fragment carrying the 2-nitrosophenyl ketone group.

4. *Synthesis and Photolysis of an Oligodeoxynucleotide Hairpin Carrying a Photolabile Linker at the Apex of the Loop.* Hairpin oligodeoxynucleotide sequence B, 5′-d(CTCAATGACTCGTT-**7**-TTCGAGTCATTGAGTCATTTTT)-3′, was prepared. The oligonucleotide has a self-complementary sequence of 15 base pairs linked by a tetrathymidine loop. In the middle of the loop, there is the photolabile 2-nitrobenzyl group (*cf.* **7**). This hairpin was designed to be immobilized to SiO_2 surfaces

in forthcoming experiments. For this reason, there are five thymidines at the 3′-end that will be used as a spacer to separate the hairpin from the surface.

After the assembly of the sequence and ammonia deprotection, the resulting oligonucleotide with the dimetoxytrityl (DMT) group was purified by RP-HPLC and characterized by mass spectrometry. During HPLC purification, a minor peak (20% of the desired product) was observed corresponding to an extra oligonucleotide sequence with the DMT group. Mass spectrometry showed that the minor DMT-oligonucleotide sequence was a hydrolysis product of the desired compound near the photolabile site (*Fig. 2*). This indicates that the photolabile linker is not totally stable under ammonia-deprotection conditions, but the desired full-length compound is easily separated from the hydrolysis compound. Photolysis was performed as described for the short oligonucleotide sequence, leading to similar results (*Fig. 4*). Most of the starting material disappeared after 30 min. This time, a small peak was observed after the starting hairpin that was assigned to the 5′-fragment with the rest of the photolabile group. Denaturing gel electrophoresis confirmed the fragmentation of the hairpin and the formation of two bands of 14 and 24 bases.

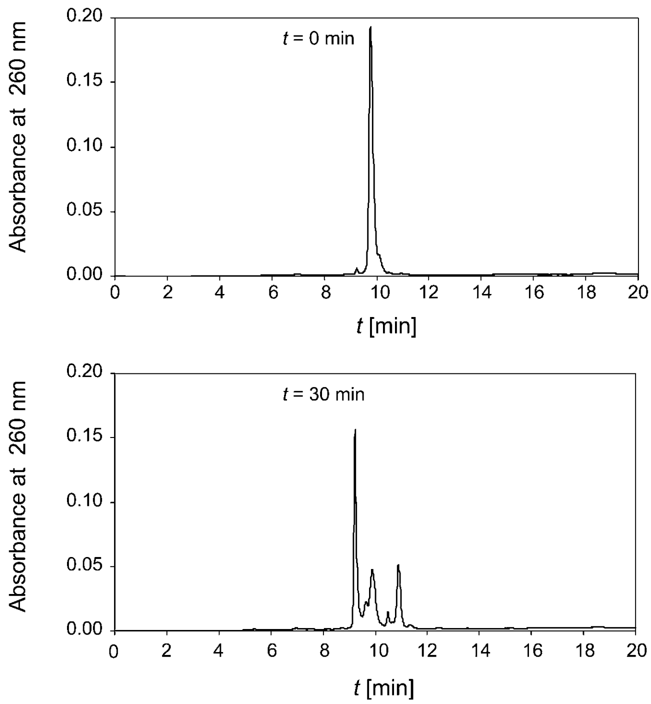

Fig. 4. *Photolysis of oligonucleotide hairpin B.* HPLC Profiles of samples at the start ($t = 0$) and after 30 min of photolysis.

Melting curves of the hairpin oligodeoxynucleotide (sequence B) with and without the presence of its complementary sequence (sequence C) were performed before and after photolysis. The hairpin shows a single duplex-to-random-coil transition with a melting temperature at 75° (*Fig. 5,a*). The transition at 75° was also the major

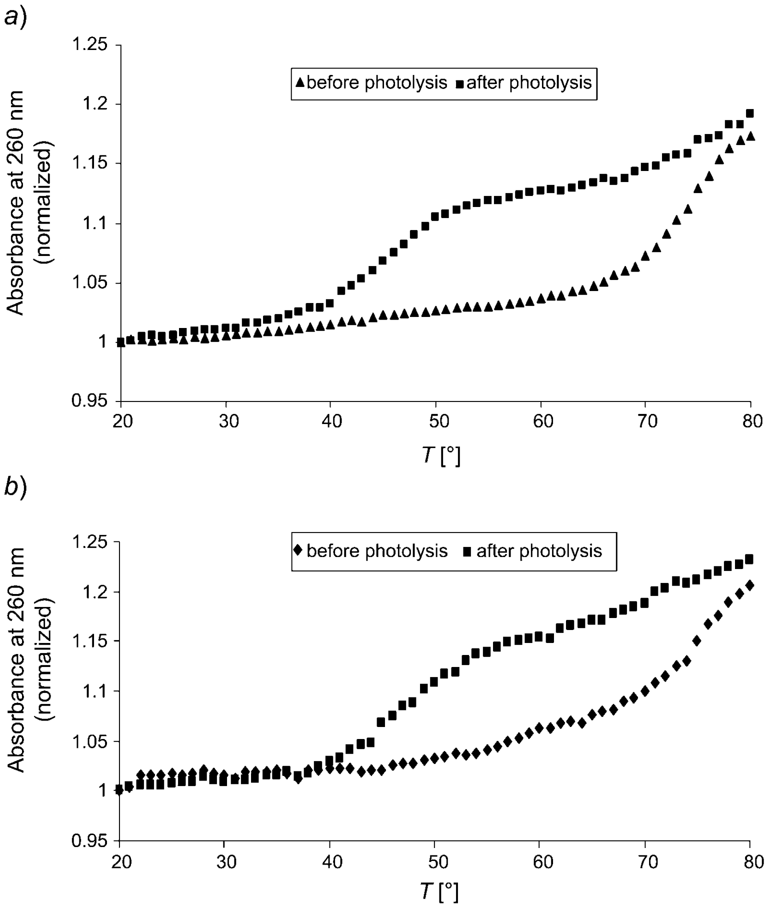

Fig. 5. *Melting curves of oligonucleotide hairpin B. a*) Hairpin alone before and after photolysis; *b*) hairpin in the presence of complementary oligodeoxynucleotide (sequence C) before and after photolysis. Conditions: 0.15M NaCl, 1 mM $MgCl_2$, and 10 mM Na_3PO_4 pH 7.13.

transition of the hairpin in the presence of an equimolar amount of its complementary sequence (*Fig. 5,b*).

After 30 min of photolysis, the melting curve was repeated. This time, the transition was observed at a lower melting temperature ($T_m = 50°$, *Fig. 5,a* and *b*), indicating that the intramolecular duplex was more stable than the intermolecular duplex. We are now examining the use of this difference in stability to fabricate patterns on surfaces. To this end, we plan to immobilize oligodeoxynucleotide hairpins carrying photolabile groups on surfaces and create patterns of double-stranded and single-stranded DNA sequences by photolysis. Hybridization with complementary oligodeoxynucleotide conjugates is expected to allow selective deposition of nanomaterials in the areas of single-stranded DNA resulting from the photolysis.

Conclusions. – We have demonstrated that photolabile 2-nitrobenzyl groups can be introduced into the middle of peptide and oligodeoxynucleotide derivatives, leading to stable derivatives that can be purified and characterized according to standard solid-phase synthesis protocols. Photocleavage of these compounds in aqueous solutions is achieved in minutes or in hours depending on the presence or absence of a Me group in the benzylic position. After photolysis, the moiety of the molecule carrying the resulting NO derivative is not easily found, probably due to further reactions of the resulting NO derivative [5][14]. In contrast, the moiety of the molecule without the NO derivative is clearly observed and identified.

An oligodeoxynucleotide hairpin carrying a photolabile group in the loop has been prepared. The modified hairpin has a very stable double-stranded form, as evidenced by melting experiments. In the presence of the complementary strand, the modified hairpin maintains the entropy-favored intramolecular form, as judged by the analysis of the melting curve. Photolysis of the hairpin generates two single-stranded molecules that have a lower melting temperature than the hairpin.

These results set the basis for the use of photolabile DNA hairpins in the manufacture of patterned surfaces by photolithography. Further work in this direction is in progress.

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Experimental Part

General. Phosphoramidites and ancillary reagents used during oligodeoxynucleotide synthesis were from *Applied Biosystems* (*PE Biosystems Hispania S.A.*, Spain). The photolabile phosphoramidite **8** was from *Link Technologies* (*Link Technologies Ltd.*, Scotland). The Fmoc-amino acids were from *Novabiochem* (*Novabiochem GmbH*, Switzerland). The rest of the chemicals were purchased from *Aldrich*, *Sigma*, or *Fluka* (*Sigma-Aldrich Química S.A.*, Spain).

4-{[(Trifluoroacetyl)amino]methyl}benzoic Acid (**2**). *4-(Aminomethyl)benzoic acid* (**1**; 3 g, 20 mmol) was reacted with CF_3COOEt (4.2 ml) in 20 ml of EtOH containing 4.2 ml of Et_3N at r.t. overnight [13]. The mixture was concentrated to dryness, and the resulting residue was dissolved in hot H_2O (70 ml). The product precipitated on cooling, and a few drops of CF_3COOH (TFA) were added, which induced more abundant precipitation. The desired product was isolated by filtration to yield 4.37 g (18 mmol, 90%) of **2**. White solid. Physical and spectroscopic properties were as described in [12][13].

2-Nitro-{[(trifluoroacetyl)amino]methyl}benzoic Acid (**3**). Compound **2** (18 mmol) was dissolved in 95% conc. H_2SO_4 (60 ml), and the soln. was cooled on ice. To the cooled soln., a mixture of conc. H_2SO_4 (2 ml) and 68% HNO_3 (1.6 ml) was added dropwise. After the addition, the mixture was allowed to warm to r.t. The resulting mixture was poured over 500 g of crushed ice. The resulting precipitate was filtered to yield 5.3 g (18 mmol) of **3** as a solid that was used without further purification. Physical and spectroscopic properties were as described in [12][13].

*4-[({[(9*H*-Fluoren-9-yl)methoxy]carbonyl}amino)methyl]-2-nitrobenzoic Acid* (**5**). Compound **3** (0.2 g, 0.68 mmol) was dissolved in dioxane/30% aq. NH_3 1:1 (10 ml), and the resulting soln. was stirred overnight at r.t. The resulting soln. was concentrated to dryness to yield a brown residue, **4**, that was used without further purification.

The residue was dissolved in dioxane/10% aq. Na_2CO_3 1:1 (20 ml). The resulting mixture was cooled in an ice bath, and 100 mg of *N*-hydroxysuccinimyl (9*H*-fluoren-9-yl)methoxycarbonate (Fmoc-OSu) dissolved in dioxane (10 ml) was added. The mixture was stirred at 4° for 45 min and allowed to warm to r.t. After 1 h of magnetic stirring, H_2O (30 ml) was added, and the mixture was treated with Et_2O (3 × 20 ml). The aq. layer was concentrated to half of its volume, and the resulting soln. was cooled in an ice bath. The cooled soln. was acidified to pH 2 with 32% aq. HCl soln. A white precipitate is formed that was collected by filtration to yield 184 mg (0.44 mmol, 65%) of a white solid. Physical and spectroscopic properties were as described in [12].

Peptide Synthesis. The peptide sequence H-^{N}Tyr-Ala-**6**-Phe-Lys-Gly^{C}-OH was prepared using solid-phase methodology and Fmoc-amino acids. Tyrosine was protected with the *t*-Bu group and lysine with the (*tert*-butoxy)carbonyl group. The peptide was assembled on polystyrene support functionalized with Fmoc-Gly from commercial sources (Fmoc-Gly-*Wang*-polystyrene [15]; 0.51 mmol/g, 50 mg, 25 μmol). The peptide chain was elongated in DMF (0.5 ml) using fivefold excess of the Fmoc-amino acid and fivefold excess of (benzotriazol-1-yl)-*N*-oxytris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) and tenfold excess of $EtN(i-Pr)_2$ for 1 h. After the assembly of the sequences, the support was treated with a soln. of TFA/H_2O 95 : 5 (2 ml) for 3 h. The support was removed by filtration, and the resulting soln. was treated with Et_2O (50 ml). A white precipitate was isolated by filtration. The resulting product was purified by RP-HPLC, and the desired product was obtained as the major component (M_r 763.4; calc. 762.9).

Oligodeoxynucleotide Synthesis. Oligodeoxynucleotide sequences A (5′-d(CCCCC-**7**-TTTT)-3′), B (5′-d(CTCAATGACTCGTT-**7**-TTCGAGTCATTGAGTCATTTTT)-3′), and C (5′-d(TGACTCAATGACTCG)-3′) were prepared using solid-phase methodology and 2-cyanoethyl phosphoramidites as monomers. The photolabile phosphoramidite (PC spacer-CE phosphoramidite, *Link Technologies*) was used to introduce the photolabile function. The syntheses were performed on an *Applied Biosystems Model 3400* DNA synthesizer. After the assembly of sequences, ammonia deprotection was performed either for 2 h at r.t. (sequence A) or overnight at 55° (sequence B). Oligonucleotides were purified by RP-HPLC. HPLC Solns. were as follows: solvent *A:* 5% MeCN in 100 mM $Et_3N \cdot OAc$ (pH 6.5) and solvent *B:* 70% MeCN in 100 mM $Et_3N \cdot OAc$ (pH 6.5); columns: *Nucleosil 120C18* (10 μm, 200 × 10 mm); flow rate: 3 ml/min. Conditions *A* (DMT on): 20 min linear gradient from 15–80% *B.* Conditions *B* (DMT off): 20 min linear gradient from 0–50% *B.*

MS: Sequence A (MALDI): found 2942.8 ($[M-H]^-$); anal. calc. for $C_{98}H_{130}N_{25}O_{63}P_9$: 2943.5). In addition to the expected mass, we observed two peaks corresponding to the fragmentation of the oligodeoxynucleotide (T_4; found 1233.5; anal. calc. for $C_{40}H_{49}N_8O_{29}P_4$: 1229.2 and C_5-photolabile group, found 1710.4; anal. calc. for $C_{58}H_{71}N_{17}O_{34}P_5$: 1704.3). Yield: 7 O.D. Units at 260 nm.

MS: Sequence B (MALDI): found 11336.7 ($[M-H]^-$); anal. calc. for $C_{366}H_{465}N_{120}O_{230}P_{36}$: 11334.9. In addition to the expected mass, we observed two peaks corresponding to the fragmentation of the oligodeoxynucleotide (3′-fragment, found 6804.7; anal. calc. for $C_{217}H_{277}N_{71}O_{140}P_{22}$: 6798.1 and 5′-fragment-photolabile group, found 4545.85; anal. calc. for $C_{149}H_{189}N_{49}O_{90}P_{14}$: 4537.8). Yield: 33 O.D. units at 260 nm.

In addition to the expected product, a minor product corresponding to an oligodeoxynucleotide sequence with a DMT group was also observed. This was identified as the product of the hydrolysis or ammonolysis of the amide present in the photolabile compound (M_r 4385).

Photolysis of Peptide and Oligodeoxynucleotides. Aq. solns. (2 ml) of peptide (0.9 mM) or oligodeoxynucleotide (0.07 mM) were photolyzed with a *Black Eye* lamp (340 nm) at r.t. Aliquots of 0.2 ml were taken at different time points (10, 30, 60, and 120 min, resp.) and analyzed by HPLC. The peptide solns. were analyzed by HPLC/MS. HPLC Solns. were as follows. Solvent *A:* 0.1% HCOOH in H_2O and solvent *B:* 0.07% HCOOH in MeCN; columns: *Spherisorb C18* (5 μm, 100 × 5 mm); flow rate: 1 ml/min. Conditions *A:* 10 min linear gradient from 0–50% *B. Fig. 1* shows the HPLC profile of samples taken at the start and after 2 h. The starting material (t_R 4.6 min, M_r 763.4) disappeared, and the fragment TyrAla was found at t_R 2.4 min (M_r 272.1 ($[M+Na^+-H^+]$); calc. 251.2). The oligodeoxynucleotide solns. were analyzed by HPLC as described in the purification of oligodeoxynucleotides (conditions *B*). *Figs. 3* and *4* show the HPLC profile of samples taken at the start of the photolysis and after 30 min. In the photolysis of sequence A, a new peak at 9 min was observed and identified as T_4 by HPLC analysis of the enzyme digestion of the product (snake venom phosphodiesterase and alkaline phosphatase [16]) that gave thymidine as single compound. The product containing five cytosines and the photolabile group was not isolated. In the photolysis of oligodeoxynucleotide sequence B, a small peak was observed eluting after the hairpin, which was assigned to the fragment at 5′ with the rest of the photolabile groups. Denaturing gel electrophoresis confirmed the fragmentation of the hairpin, and the formation of two bands of 14 and 24 bases.

Melting Studies. Oligodeoxynucleotide hairpin B with or without an equimolar amount of its complementary sequence (sequence C) was dissolved in an aq. buffer containing 0.15M NaCl, 1 mM $MgCl_2$, and 10 mM Na_3PO_4, pH 7.13, and placed in a spectrophotometer equipped with temp. controller. Melting experiments were performed in the temp. range from 15 to 80° with a linear temp. ramp of 0.5°/min. Absorbance spectra were recorded every 1°. Each sample was allowed to equilibrate at the initial temp. for 30 min before the melting experiment was started.

HPLC Analysis of the soln. of the hairpin alone obtained after the melting experiment showed the presence of 15% of the photolyzed hairpin. This partial photolytic cleavage is due to the UV light used during the melting experiment. A second melting experiment with the same sample showed a small transition at *ca.* 50° due to the presence of the photolyzed hairpin produced during the first melting experiment.

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