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Antiviral effect of ribonuclease conjugated oligodeoxynucleotides targeting the IRES RNA of the hepatitis C virus

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ABSTRACT

Hepatitis C virus (HCV) translation initiation is mediated by a highly structured and conserved RNA, termed the Internal Ribosome Entry Site (IRES), located at the 5′-end of its single stranded RNA genome. It is a key target for the development of new antiviral compounds. Here we made use of the recently developed HCV cell culture system to test the antiviral activity of artificial ribonucleases consisting of imidazole(s) linked to antisense oligodeoxynucleotides targeting the HCV IRES. Results from the cell culture model indicate that the naked antisense oligodeoxynucleotide displayed an efficient antiviral activity. Despite the increased activity observed with the addition of imidazole moieties when tested with the cell-free system, it appears that these improvements were not reproduced in the cellular model.

Chronic hepatitis C virus (HCV) infection affects more than 170 million people worldwide. The standard treatment consists of a combination of pegylated interferon-alpha and ribavirin. However, although such treatment is somewhat successful on HCV genotypes 2 and 3, it is less effective with genotypes 1 and 4. This lack of activity, the existence of some severe adverse side effects, contraindications and the emergence of HCV drug resistance limit efficacy and urge for the development of new therapies.[1] Among the therapeutic targets of HCV, its genomic RNA 5′-untranslated region (5′-UTR) appears to be a good candidate for molecular therapy. HCV has evolved an unusual mode of translation initiation that is mediated by a highly structured RNA, termed Internal Ribosome Entry Site (IRES), located at the 5′-end of its 9.5-kb nucleotide RNA genome.[2] Contrary to the canonical cap-dependent initiation of most eukaryotic mRNAs, the HCV IRES can recruit 40S ribosomal subunits independently of either an m7G-cap structure at the mRNA 5′-end or the scanning of the viral mRNA. This allows simplified and efficient translation of its genomic RNA.[3] The conserved secondary structure of the HCV IRES comprises ~340 nucleotides, encompassing three main structural domains II to IV (Fig. 1).

The two large helical domains termed II and III are bridged by a pseudoknot to domain IV that is a short stem-loop. Domain III associates with the outer surface of the 40S ribosomal small subunit platform and is required for the high affinity interaction of the IRES with the latter, while domain II contacts the head of the ribosomal subunit. Both structural domains interact with the multisubunit initiation factor eIF3.[4] Among the complex structured regions of the HCV IRES, subdomain IIId adopts the well characterized loop E motif. It plays an important role during the early steps of ribosome recognition and therefore represents a potential target for structure-based drug design.[5]Accordingly, aptamers targeted to domain IIId were generated by in vitro selection (SELEX) and tested both in a cell-free system and in cell culture. This was done using transient transfection and a reporter mRNA where translation was under the control of the HCV IRES.[6] Antisense molecules targeting the IRES can only prevent the translation of the HCV polyprotein. In order to improve their activity, we recently developed a strategy which combines the binding affinity of the antisense oligodeoxynucleotides (***AS-ODNs***) with the capability of artificial ribonucleases to cleave the RNA at specific locations.[7] Conjugates were synthesized by coupling imidazole moieties, known to mimic RNase A catalytic centers,[8] to ***AS-ODNs*** whose sequences were inspired by RNA aptamers targeting domain IIId.[6] Therefore, these compounds should disrupt the HCV life cycle by preventing translation and replication. In the present study we investigated the toxicity and antiviral activity of the most efficient of these

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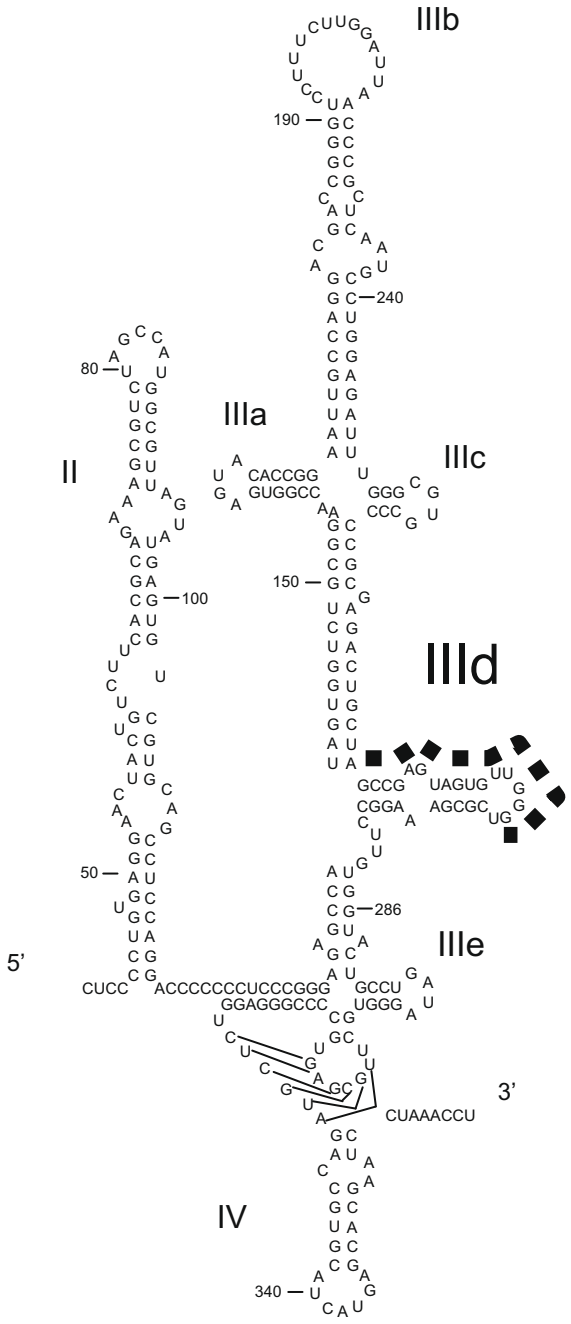


Figure 1. Secondary structure of the IRES of the HCV genomic RNA. The predicted binding site of the 17-mer antisense oligodeoxynucleotide ***AS-ODN*** used in this study within the domain IIId sequence is indicated (dashed).

compounds in vitro by making use of the recently developed propagation system of infectious HCV particles in cell culture (HCVcc).[9] This system reproduces the entire life cycle of the virus in a hepatic cell line. In order to improve their activity, a new original compound containing two imidazole moieties instead of one was also designed, produced and assessed.

To facilitate screening of the compounds, we used a modified HCVcc with a genome that contains a luciferase reporter gene.[10] The naked antisense DNA (***AS-ODN***) and non specific DNA (***NS-ODN***) sequences used for this study were as follows: **ACCCAACAC-TACTCGGC** and **CAACCCTAGCCCGTCAA** respectively. In the first series of experiments, the naked ***AS-ODN*** or the ***AS-ODN*** coupled with either 5′-imidazole or with 3′-imidazole[7] (Fig. 2) were microporated into Huh7.5.1 cells 17 h prior to infection with HCVcc. The ability of the oligodeoxynucleotides to interfere with establishment of HCV replication was then measured relative to that obtained with the ***NS-ODN***, consisting of a randomly scrambled sequence of the ***AS-ODN***. The growth and viability of the cells was not affected by any of the oligodeoxynucleotides.[11]

The presence of the naked ***AS-ODN***, the 5′-imidazole or the 3′-imidazole conjugates within the cells reduced, in a dose-dependent manner and up to 50%, the level of viral replication established in Huh7.5.1 after their infection with HCVcc. The maximum antiviral activity was achieved at a 100 nM concentration after 20 h of infection. No further inhibition was achieved by increasing the concentration of oligodeoxynucleotides to 1000 nM (Fig. 3). Unlike the results obtained with the cell-free system,[7] the addition of an imidazole moiety to either the 3′ or 5′ end of the naked AS-ODN did not result in an increase in their antiviral properties when tested on the HCVcc propagation system. This result suggested that in cells, the process of cleavage by a single imidazole was not the main factor responsible for activity as was previously observed in the cell-free system.

To better mimic the catalytic center of RNase A, we prepared a novel compound containing two imidazole moieties. The design and synthesis of such bifunctional catalysts have received considerable attention,[12] since residues His 12 and His 119 in the catalytic site of RNase A serve respectively as base and acid catalysts and the protonated side chain of Lys-41 stabilizes the pentacoordinated phosphorous intermediate during transition state.[13] The bis-imidazole-containing ***AS-ODN*** was synthesized by standard protocol from the acid **8** (Scheme 1). First, urocanic acic **1** was esterified with methanol[14] and protected as its *N*-dimethylsulfamoyl derivative **3.** Reduction with diisobutylaluminium hydride and treatment with thionyl chloride afforded the allylchloride **5**.[15] Sodium salt of diethylmalonate was then alkylated with two equivalents of **5** to give the diester **6**, which was hydrogenated in the presence of Pd on charcoal. Deprotection and decarboxylation were simultaneously achieved by heating at reflux in 6 M HCl. Analysis of the reaction mixture revealed the concomitant formation of dimethylamine hydrochloride. Final purification by ion exchange chromatography on Dowex H^+ 50W8 gave the desired compound **8**.[16] The coupling reaction was carried out in the presence of PyBOP, HOBT and N-methylmorpholine in DMF at the 5′-end of the oligodeoxynucleotide, since the 5′ mono-imidazole displays the highest activity in vitro.[7,17]

First, the antiviral activity of this bis-imidazole compound was tested in a cell-free system and compared to the naked antisense and to the mono-imidazole compounds. The specific cleaving activity of the bis-imidazole coupled to the ***AS-ODN*** was also confirmed by using an in vitro transcribed and labeled HCV IRES RNA fragment. The inhibitory effects of the bis-imidazole conjugate on HCV IRES driven translation of a *Renilla* luciferase reporter mRNA were also confirmed by using a reticulocyte based translation assay. However, despite the use of two imidazole groups within the same molecule, a similar level of viral translation inhibition was achieved as compared to the 5′-mono imidazole compound, whatever the concentration used (not shown).

We then decided to investigate the action of this modified oligodeoxynucleotide in a more physiological system by using hepatic cell cultures infected with HCVcc. The optimal concentration determined in the previous experiments (100 nM) was the concentration chosen to test the bis-imidazole conjugate. As observed with the cell-free approach, there was no improvement in the antiviral activity of the ***AS-ODN*** when coupled to two imidazoles instead of one. Indeed, whatever the compound (naked or coupled ***AS-ODN***) previously introduced into the cell, the establishment of viral replication after HCVcc infection was inhibited by approximately half (Fig. 4).

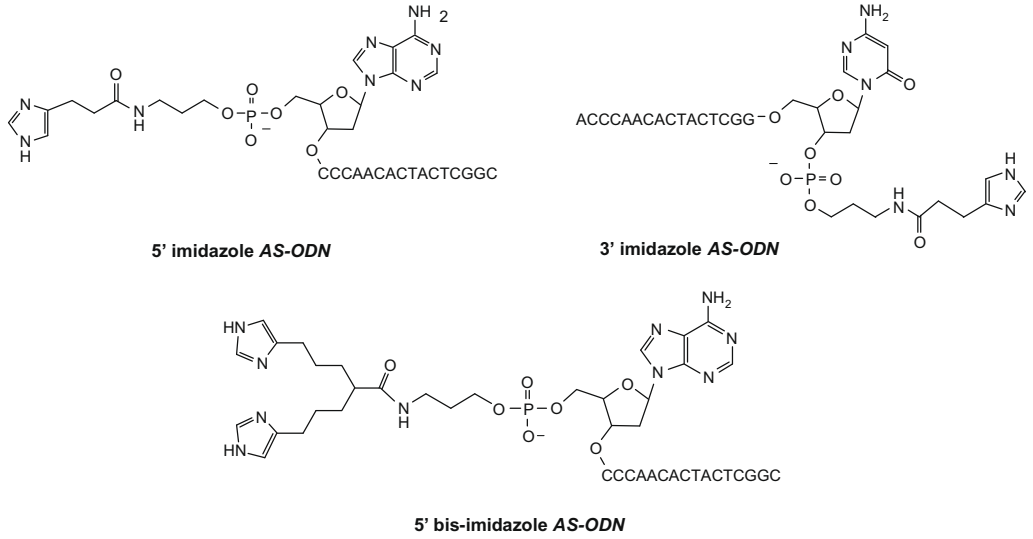


Figure 2. Sequences of ribonuclease conjugated oligodeoxynucleotides used in this study.

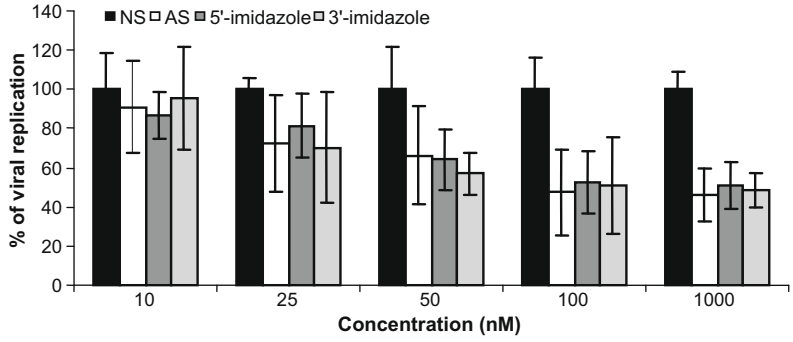
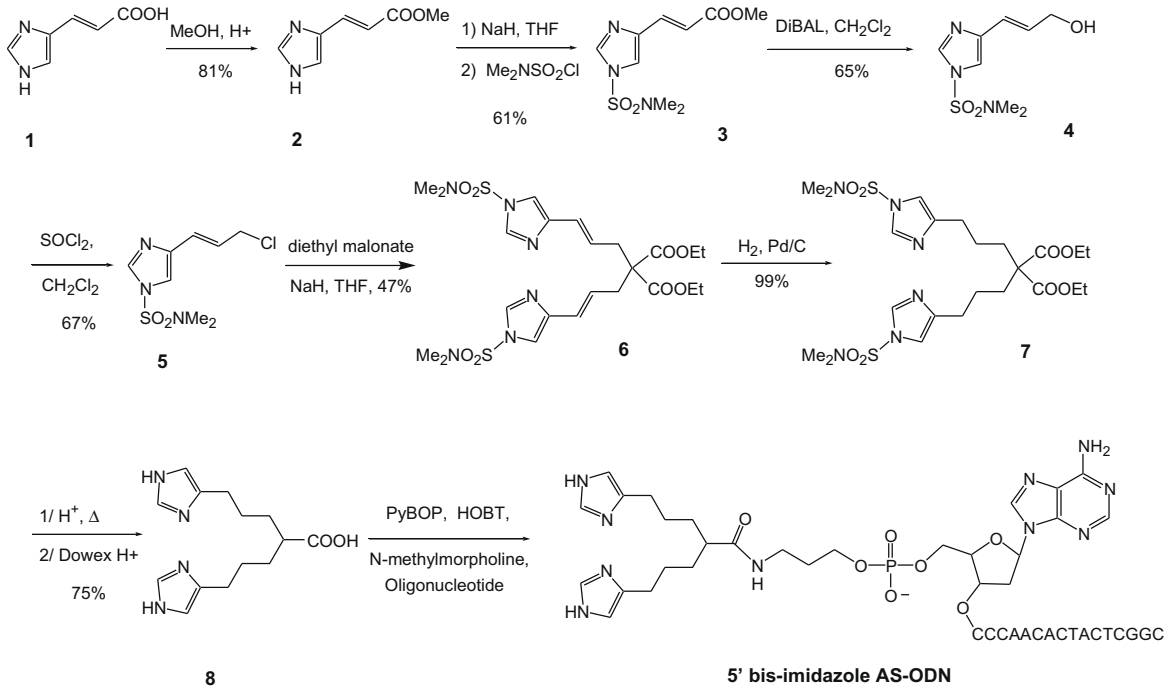


Figure 3. Effect of naked or mono-imidazole coupled oligodeoxynucleotides on cell infection by HCVcc. Huh7.5.1 cells were electroporated with either the non-specific ODNs (***NS-ODN***), the naked antisense ODN (***AS-ODN***), the 5′-imidazole or the 3′-imidazole conjugated ***AS-ODN*** at different concentrations prior to infection with HCVcc. Experiments were performed in triplicate and error bars represent the standard deviation of the mean. Results were normalized against cell number as determined by WST-1 Cell Proliferation Assay (Roche). Values are expressed as percentages of the NS controls.



Scheme 1. Synthesis of the bis-imidazole conjugated ***AS-ODN***.

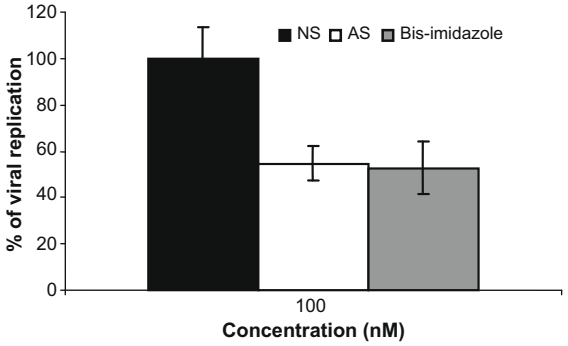


Figure 4. Effect of the bis-imidazole oligodeoxynucleotide conjugate on cell infection by HCVcc. Huh7.5.1 cells were electroporated with either the non-specific ODNs (***NS-ODN***), the naked antisense ODN (***AS-ODN***) or the newly synthesized oligodeoxynucleotide, 5′bis-imidazole, at a concentration of 100 nM. Experiment was performed in triplicate and error bars represent the standard deviation of the mean. Results were normalized against cell number as determined by the WST-1 Cell Proliferation Reagent (Roche). Values are expressed as percentages of the NS controls.

One hypothesis for this lack of improvement might be the extended time the oligodeoxynucleotides were present in the cells before addition of HCVcc (17 h). Such a prolonged infection period may lead to a partial inhibition of the cleaving properties of the compounds. Infecting cells containing oligodeoxynucleotides at time-points of less than 17 h was attempted. However, this proved to be toxic to the cells, most likely because they have not recovered from transfection.

We therefore decided to test our oligodeoxynucleotide panel by infecting cells with HCVcc prior to transfection. This would allow the oligodeoxynucleotides to act on the IRES immediately, rather than after a 17 h delay where degradation may take place. The trypsination of Huh7.5.1 cells after infection with our recombinant HCVcc decreased the proportion of HCV replicating cells. Since it is a step required for microporation, we therefore chose to transfect Huh7.5.1 cells after HCVcc infection using a lipid reagent.

We initially confirmed that the antiviral activity of the ***AS-ODN*** could be achieved using lipofection. Thus, ***AS-ODN*** was transfected at a final concentration of 100 nM, the concentration which gives the maximum levels of inhibition with microporation. After a 17 h incubation period, cells were infected with HCVcc. Using lipofection yielded similar inhibition results to those obtained with microporation. As found previously, there was no cytotoxic effect of the oligodeoxynucleotides. Once we had confirmed that transfection of the oligodeoxynucleotides was possible using lipofection, we then tested the action of the oligodeoxynucleotides on Huh7.5.1 cells that were already infected with HCVcc. As seen in Figure 5, ~10-fold higher concentration of oligodeoxynucleotides was required to detect antiviral activity when HCV replication was already established in cells. A maximum of 50% viral inhibition was achieved at a concentration of 1000 nM. The limited level of inhibition achieved in these experimental conditions may be partly ascribed to the eventual competition of the compounds with cellular factor(s) interacting with the same region of the HCV RNA. It may also be due to the higher level of IRES RNAs to target. However, no increase in inhibition was achieved with the addition of imidazole or bis-imidazole to the naked ODN sequence.

Noteworthy, the same level of inhibition of viral replication was achieved when comparing a 5′-end imidazole linked 2′-*O*-Me-RNA oligonucleotide to its naked counterpart (data not shown).

Therefore, despite the increased activity observed with the addition of imidazole moieties when tested with a cell-free translation assay, it appears that this improvement is not observed in infected Huh7.5.1 cells. We cannot rule out that factor(s) present in human hepatic cells, but absent from rabbit reticulocytes, could outweigh or mask the cleavage sites targeted by the imidazole moieties. Therefore, in order to improve their intrinsic effects, stabilized in vivo compounds will be developed. A promising approach could be the use of short peptide nucleic acids (PNA) that have recently been shown to display stronger activity than DNA or RNA when targeting HCV IRES driven translation.[18] Furthermore, the use of a peptidic bond to bind the RNAse A mimic to ***AS-ODNs*** makes this system adaptable and easily interchangeable with other cleaving agents. The tool developed here will now be adapted to target other domains of the genomic RNA of HCV.

Acknowledgments

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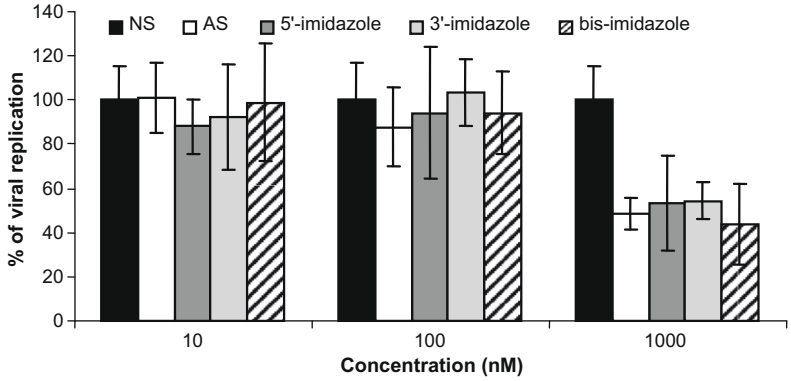

Figure 5. Effect of oligodeoxynucleotides on cells already infected with HCVcc. Huh7.5.1 cells were initially infected with HCVcc. After a 17 h incubation, cells were transfected with either the non-specific ODN (***NS-ODN***), the naked antisense ODN (***AS-ODN***), the 5′-imidazole, the 3′-imidazole or the 5′bis-imidazole at different concentrations. Experiments were performed in triplicate and error bars represent the standard deviation of the mean. Results were normalized against cell number as determined by WST-1 Cell Proliferation Reagent (Roche). Values are expressed as percentages of the NS controls.

Supplementary data

Supplementary data (cell culture, production and titration of HCVcc, Huh7.5.1 cell transfection methods and cell viability and luciferase assays) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.04.139.

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16. *5-(1H-imidazol-4-yl)-2-[3-(1H-imidazol-4-yl)propyl]pentanoic acid* **8**: 200 mg of **6** (0.34 mmol) were dissolved in 10 mL of methanol. 21 mg of Pd/C 10% were added to the solution. After 20 h of stirring under 45 bars of dihydrogen, the mixture was filtered and the filtrate concentrated to dryness to give 200 mg of **7** as a clear yellow oil (99% yield). The product was used without further purification in the next step. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.24 (t, 6H, J = 7.1 Hz), 1.50–1.56 (m, 4H), 1.92–1.98 (m, 4H), 2.60 (t, 4H, J = 7.4 Hz) 2.86 (s, 6H), 4.18 (q, 4H, J = 7.1 Hz), 7.00 (s, 2H), 7.85 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.4, 143.1, 136.1, 113.7, 61.2, 57.1, 38.1, 31.6, 27.9, 23.1, 14.0. 200 mg of crude **7** were dissolved in 10 mL of 6 M HCl. The solution was refluxed for 4 days before being allowed to cool to room temperature. Evaporation to dryness afforded an oily residue. ^{1}H NMR (300 MHz, D_2O) δ 1.48–1.53 (m, 8H), 2.28–2.39 (m, 1H), 2.59–2.73 (m, 4H), 7.07(s, 2H), 8.42 (s, 2H). ^{13}C NMR (75 MHz, D_2O) δ 180.9, 133.4, 132.6, 115.1, 44.8, 30.6, 25.4, 23.5. Dimethylamine hydrochloride was identified in the crude mixture by its NMR data. ^{1}H NMR (300 MHz, D2O) δ 2.59 (s, 6H). ^{13}C NMR (75 MHz, D_2O) δ 34.5 (same values than those obtained with an authentic sample). The residue was then dissolved in 15 mL of distilled water before purification by ion-exchange column chromatography Dowex H^+ 50W8. The product was eluted with concentrated ammonium solution. The fractions were pooled, concentrated and lyophilized to give 70 mg of **8** as a very hygroscopic clear brown solid (75% yield). ^{1}H NMR (300 MHz, D2O) δ 1.29–1.34 (m, 4H), 1.43–1.53 (m, 4H), 2.06–2.16 (m, 1H), 2.56 (t, 4H, J = 7.0 Hz), 6.98 (br s, 2H), 8.17 (br s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 185.1, 135.1, 133.5, 115.8, 48.5, 31.9, 26.2, 24.1. ESI (MeOH) HRMS $[M+H]^+$ calculated for $C_{14}H_{21}N_4O_2$ 277.16645; found. 277.1658.
17. The phosphodiester oligodeoxynucleotide conjugate, prepared as previously described (Ref. 7), was analyzed and purified by reverse phase HPLC on Uptisphere ODB C18-5µ columns (Interchim, France) using Agilent 1200 system (Agilent). The purified oligodeoxynucleotide conjugate was analyzed by MALDI-TOF mass spectrometry using an UltraFlex Mass Spectrometer (Bruker Daltonics, Germany). The measured mass was in accordance to the theoretically calculated value.
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