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ORIGINAL ARTICLE

Synthesis of a diaminopropanoic acid-based nucleoamino acid and assembly of cationic nucleopeptides for biomedical applications

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Abstract In this work, we report a synthetic approach to a Fmoc-protected nucleoamino acid, based on L-diaminopropanoic acid, carrying the DNA nucleobase on the alpha-amino group by means of an amide bond, suitable for the solid-phase synthesis of novel nucleopeptides of potential interest in biomedicine. After ESI–MS and NMR characterization this building block was used for the assembly of a thymine-functionalized nucleopeptide, composed of nucleobase-containing L-diaminopropanoic acid moieties and underivatized L-lysine residues alternated in the backbone. Circular dichroism studies performed on the cationic nucleopeptide and adenine-containing DNA and RNA molecules suggested that the thymine-containing peptide is able to interact with both DNA and RNA. In particular, a significant conformational variation in the RNA structure was suggested by CD studies. Human serum stability assays were also conducted on the cationic nucleopeptide, which was found to be highly resistant to enzymatic degradation.

Keywords Nucleopeptide · DNA · RNA · CD

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Introduction

In the last decades, several research studies have shown that the sugar phosphodiester linkage of nucleic acids can be variously modified leading to oligonucleotide analogs with interesting properties (Abramova et al. 1991; Bell and Micklefield 2009; D'Alonzo et al. 2011; Zatsepin et al. 2003). For example, an extensive work was performed by many research teams on peptide-based analogs of nucleic acids which showed in some cases a good ability to interact with natural nucleic acids by forming complexes of high thermal stability and a significant intrinsic cell-membrane permeability (Roviello et al. 2010b) or the ability to form supramolecular structures (Petraccone et al. 2005). In particular remarkable cell-permeability (Katritzky and Narindoshvili 2008; Dragulescu-Andrasi et al. 2006) and specific and stable DNA binding (Calabretta et al. 2009) were some of the useful properties observed in case of positively-charged analogs with pseudopeptide backbones. Nevertheless, several attempts to use real peptides as alternative oligonucleotide linkages have also been reported, allowing for the obtainment of chiral nucleopeptides with interesting properties (van der Laan et al. 1998; Roviello et al. 2010b).

Diamino acids represent a class of natural building blocks frequently used for the construction of peptide-like analogs of nucleic acids in which they offer the possibility to anchor the nucleobases, by amidation to the side amino groups, to the main peptide backbone obtained by the oligomerization of the diamino acid moieties also in combination with other amino acids (Roviello et al. 2006, 2007, 2010c) (Fig. 1).

Taking into account all these considerations, together with the importance of the diamino acid-containing nucleopeptides, we designed and realized a novel oligonucleotide

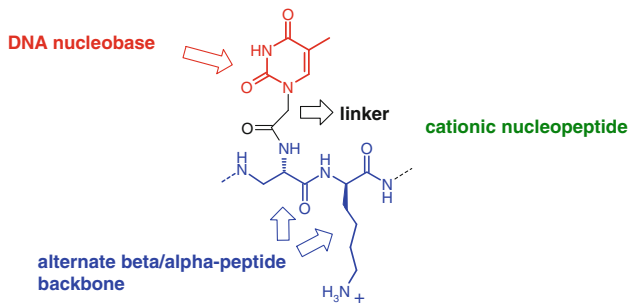


Fig. 1 Structural representation of the repeating unit of a L-diaminopropanoic-containing nucleopeptide

analog, characterized by a peptide backbone comprising both L-diaminopropanoic acid and underivatized L-lysine moieties alternated in the sequence.

In the design of this oligomer, the diamino acid moieties allow for the linkage to the DNA nucleobase by means of an amide bond to the alpha-amino moiety, whereas the L-lysine residues confer a positive charge to the oligonucleotide analog, characteristic of potential importance for improving the water-solubility as well as the ability to recognize the negatively-charged natural oligonucleotides. Some biological properties of this sequential nucleopeptide, such as the ability to interact with DNA and RNA, as well as the human serum stability were studied and described in the present work.

Materials and methods

Abbreviations

The following abbreviations are used: Ac_2O (acetic anhydride); Boc (*tert*-butoxycarbonyl); DCM (dichloromethane); DIEA (*N,N*-diisopropylethylamine); DMF (*N,N*-dimethylformamide); DMSO (dimethylsulfoxide); Fmoc (9-fluorenylmethoxycarbonyl); HATU (*O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate); NMP (4-methylpyrrolidone); PyBop (Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate); TCH_2COOH (thymin-1-yl acetic acid); TFA (trifluoroacetic acid); TMP (2,4,6-trimethylpyridine).

Chemicals

Boc-L-DAP(Fmoc)-OH, Fmoc-Gly-OH, Fmoc-L-Lys (Boc)-OH, HATU, PyBOP were purchased from Novabiochem. Anhydroscan DMF and NMP were purchased from LabScan. Piperidine was Biosolve. Solvents for HPLC chromatography and acetic anhydride were purchased from Reidel-de Haën. PolyA, TFA and TMP and Rink-amide resin were Fluka. dA_{12} DNA was purchased from Biomers. DCM, DIEA and TFA (for HPLC) were Romil. Deuterated DMSO, TCH_2COOH and TIS were purchased from Sigma-Aldrich.

Apparatus

^1H NMR and ^{13}C NMR spectra were recorded at 25 °C on Varian unity 600 MHz spectrometer. Chemical shifts (δ) are given in parts per million (ppm). Proton chemical shifts were referenced to residual CHD_2SOCD_3 ($\delta = 2.49$, quin) signals. ^{13}C NMR chemical shifts were referenced to the solvent (CD_3SOCD_3: $\delta = 39.5$, sept). Crude samples containing nucleopeptides were centrifuged for 4 min at 4,000 rpm (Z 200 A, Hermle). Products were analysed by LC–MS, performed on an MSQ mass spectrometer (ThermoElectron, Milan, Italy) equipped with an ESI source operating at 3 kV needle voltage and 320 °C, and with a complete Surveyor HPLC system, comprising an MS pump, an autosampler, and a PDA detector, by using a Phenomenex Jupiter C18 300 Å (5 µm, 4.6 × 150 mm) column. Gradient elution was performed (monitoring at 260 nm) by building up a gradient starting with buffer A (0.05 % TFA in water) and applying buffer B (0.05 % TFA in acetonitrile) with a flow rate of 0.8 ml/min.

Semi-preparative purifications were performed by RP-HPLC on a Hewlett Packard/Agilent 1100 series, equipped with a diode array detector, by using a Phenomenex Jupiter C18 300 Å (10 µm, 10 × 250 mm) column. Gradient elution was performed at 25 °C (monitoring at 260 nm) by building up a gradient starting with buffer A (0.1 % TFA in water) and applying buffer B (0.1 % TFA in acetonitrile) with a flow rate of 4 ml/min. Samples containing nucleopeptides (crude or purified), were lyophilized in a FD4 Freeze Dryer (Heto Lab Equipment) for 16 h.

Circular dichroism (CD) spectra were recorded at 10 °C on a Jasco J-810 spectropolarimeter, whereas, ultraviolet (UV) spectra were recorded on a UV–Vis Jasco model V-550 spectrophotometer equipped with a Peltier ETC-505T temperature controller, using a Hellma Tandem quartz cell 2 × 0.4375 cm (Rocchi et al. 1972; Krzyzanowska et al. 1998).

Synthesis of the Fmoc-protected thymine-bearing diamino acid (3)

Nβ-Fmoc-L-2,3-diaminopropanoic acid, 2 (Scheme 1)

Commercial Boc/Fmoc-protected 2,3-diaminopropanoic **1** (200 mg, 0.47 mmol) was treated with a 1:1 TFA/DCM solution (8 mL) at 50 °C under stirring. After 1 h the liquid phase was removed under vacuum. The crude material was treated with cold diethyl ether and after centrifugation a

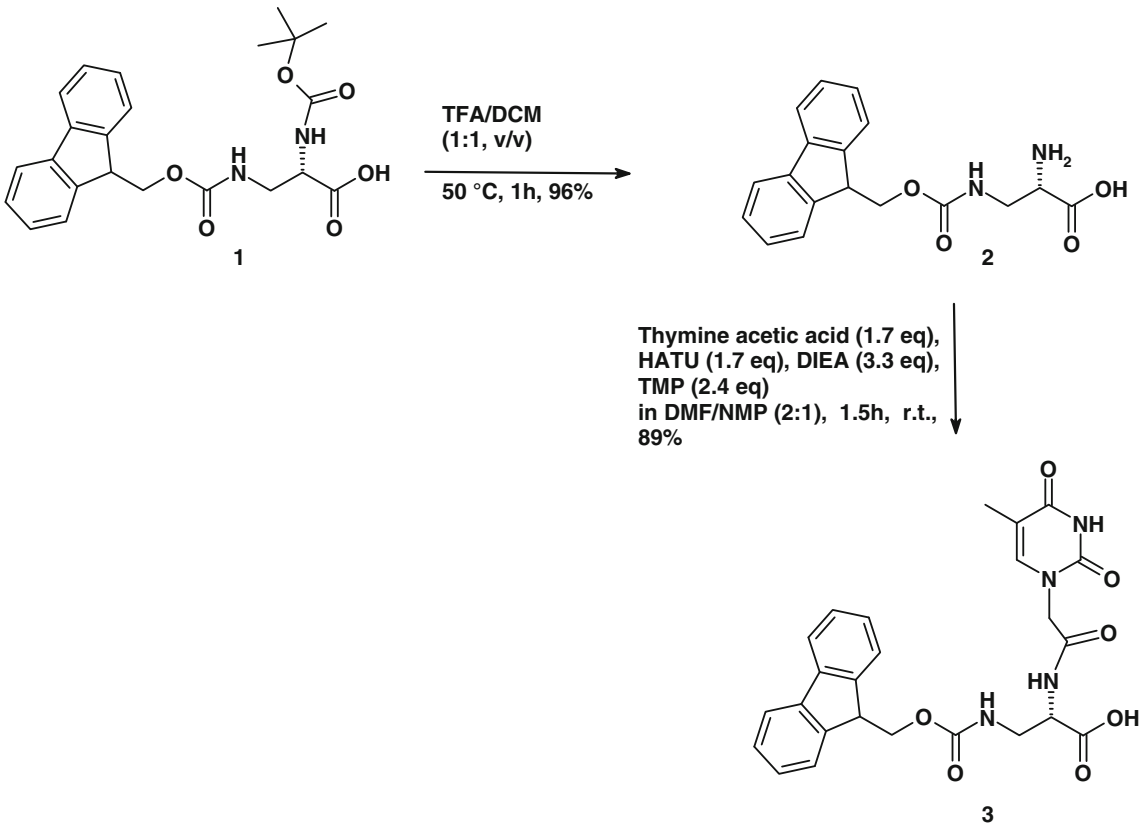
white precipitate was collected. Subsequently, after filtration this white solid was washed three times by diethyl ether and DCM, and dried under vacuum. The identity of the desired intermediate product, obtained in 96 % yield (147 mg, 0.45 mmol), was confirmed by LC–MS. ESI–MS m/z: 328.14 (found), 327.37 (expected for $[C_{18}H_{18}N_2O_4 + H]^+$); 654.81 (found), 653.73 (expected for $[2 \times (C_{18}H_{18}N_2O_4) + H]^+$); 981.63 (found), 980.09 (expected for $[3 \times (C_{18}H_{18}N_2O_4) + H]^+$).

Nβ-Fmoc-Nα-(thymin-1-ylacetyl)-L-2,3-propanoic acid, 3 (Scheme 1)

The Fmoc-protected diamino propanoic previously obtained (1 eq, 147 mg, 0.45 mmol), solved in anhydrous DMF (2 mL) and treated with DIEA (1.6 eq, 122.2 μL, 0.72 mmol) and TMP (0.7 eq, 40.3 μL, 0.30 mmol), was reacted with TCH_2COOH (1.7 eq, 141 mg, 0.76 mmol) which was previously preactivated by HATU (1.7 eq, 289 mg, 0.76 mmol) and DIEA (1.7 eq, 129 μL, 0.76 mmol)/TMP (1.7 eq, 101 μL, 0.76 mmol) in NMP (1 mL) for 2 min. After 1.5 h the solvent was removed under vacuum. The crude obtained was treated by water (50 mL) and after sonication the aqueous solution was removed by filtration from the precipitate which was obtained pure in 89 % yield (197 mg, 0.40 mmol); LC-ESIMS (Figure S.1) m/z 494.34 (found), 493.50 (expected for $[C_{25}H_{24}N_4O_7 + H]^+$); m/z 515.02 (found), 515.48 (expected for $[C_{25}H_{24}N_4O_7 + Na]^+$); m/z 984.32 (found), 985.99 (expected for $[2 \times (C_{25}H_{24}N_4O_7) + H]^+$); δ_H (600 MHz, DMSO-d6) 11.23 (1H, s, NH thymine), 8.39 (1H, d, $J = 7.8$, NH amide), 7.88 (2H, d, $J = 7.8$, aromatic protons, CH Fmoc), 7.69 (2H, t, aromatic protons, CH Fmoc), 7.43–7.29 (6H, m, aromatic protons, CH Fmoc, Fmoc-NH, CH thymine), 4.40–4.22 (6H, m, CH_2 linker, FmocCH–CH_2 and CH_α), 3.45–3.31 (2H, m, CH_2NH), 1.74 (3H, s, CH_3 thymine); δ_C (150 MHz, DMSO-d6) 171.59 (COOH), 167.19 (thymine C-4), 164.56 (CH_2CONH), 156.43 (OCONH), 151.16 (aromatic carbons, Fmoc 2C), 143.98 (thymine C-2), 142.10 (aromatic carbons, Fmoc 2C), 140.85 (thymine C-6), 127.68 (aromatic carbons, Fmoc 2CH), 127.17 (aromatic carbons, Fmoc 2CH), 125.26 (aromatic carbons, Fmoc 2CH), 120.11 (aromatic carbons, Fmoc 2CH), 108.34 (thymine C-5), 65.71 (Fmoc CH_2), 53.72 (CH_2 acetyl linker), 49.13 (CH_2 NH), 46.79 (CH_α), 41.77 (Fmoc CH), 11.82 (thymine CH_3).

Solid phase synthesis of oligomer 4

Oligomer **4** was assembled on Rink-amide MBHA resin (0.50 mmol/g, 40.0 mg) using the synthetic strategy described in Scheme 2. More particularly, after Fmoc

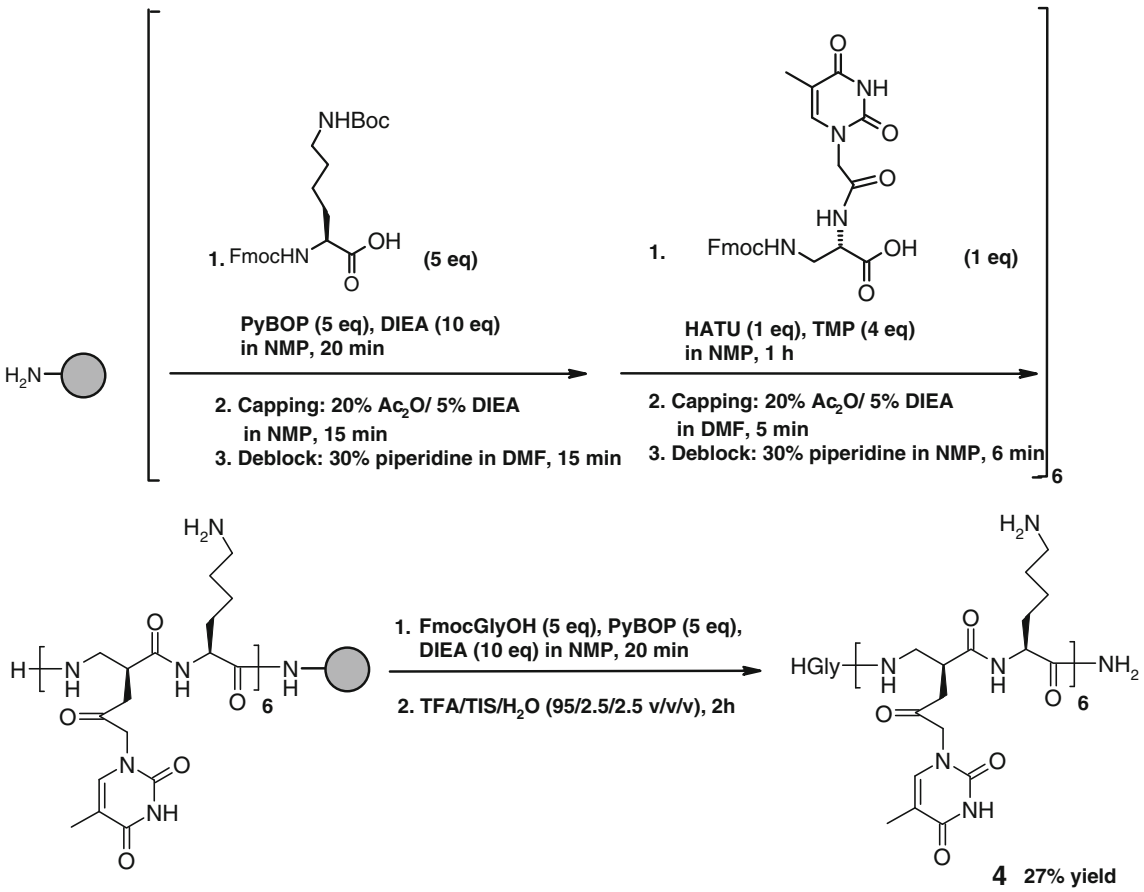


Scheme 1 Synthesis of monomer **3**

removal from the resin (by treatment with 30 % piperidine in DMF for 30 min) a mixture of Fmoc-Lys(Boc)-OH (200 μL of a 0.5 M solution in NMP, 100 μmol, 5 eq), PyBOP (200 μL of a 0.5 M solution in NMP, 100 μmol, 5 eq) and DIEA (34 μL, 200 μmol, 10 eq) was introduced in the reactor and stirred at room temperature. After 20 min the liquid phase was removed from the resin which was washed with NMP, and, subsequently, unreacted amino groups were capped by treatment with Ac_2O (20 %)/DIEA (5 %) in NMP for 15 min and Fmoc group was removed by treatment with 30 % piperidine in DMF (Deblock solution) for 15 min.

Subsequently, a mixture of Fmoc-protected nucleoamino acid **3** (200 μL of a 0.1 M solution in NMP, 20 μmol, 1 eq) and HATU (50 μL of a 0.4 M solution in NMP, 20 μmol, 1 eq) was introduced in the reactor and stirred at room temperature. 200 μl (80 μmol, 4 eq) of a TMP solution (0.4 M in NMP) were, thus, added to the mixture under stirring in 4 portions over 1 h. This condensation step was followed by capping performed with Cap solution for 5 min, whereas Fmoc-group removal was performed by treatment with Deblock solution for 6 min. Each Fmoc removal step was followed by UV Fmoc test.

The above-described procedure for the sequential introduction of the lysine and nucleoamino acid residues was repeated 6 times. Finally, a glycine unit was introduced by reaction with 450 μL of Fmoc-Gly-OH/PyBOP/DIPEA (5 eq/5 eq/10 eq) in NMP over 20 min. A 40 % overall yield was estimated by Fmoc UV test. The cleavage from the resin and deprotection of the nucleopeptide was performed by treatment with a TFA/TIS/H_2O (95/2.5/2.5 %) solution for 120 min. After precipitation with cold diethyl ether the sample solved in milliQ H_2O, was purified by RP-HPLC using a linear gradient of 10 % (for 5 min) to 30 % B in A over 30 min: $t_R = 15.1$ min; The purified product was solved in a known amount of milliQ water and quantified by UV absorption at 260 nm. The epsilon value used for the quantification of the oligomer (51600 M^{-1} cm^{-1}) was calculated by the molar extinction coefficient of thymine PNA monomer (8600 M^{-1} cm^{-1}). UV quantification of the purified product gave 5.4 μmol of product (27 % yield); ESI–MS (Figure S3) m/z: 1179.31 (found), 1179.27 (expected for $[C_{98}H_{150}N_{38}O_{31} + 2H]^{++}$); 787.38 (found), 786.52 (expected for $[C_{98}H_{150}N_{38}O_{31} + 3H]^{+++}$).



Scheme 2 Synthesis of oligomer **4**

UV and CD studies

UV and CD spectra were recorded in the 190–320 nm wavelength range using a 1 cm or 2 × 0.4375 cm (Rocchi et al. 1972; Krzyzanowska et al. 1998) Hellma quartz cell respectively. CD spectra were obtained by using the following parameters: scan speed 50 nm/min, data pitch 2 nm, band width 2 nm, response 4 s, 5 accumulations.

Serum stability assay

Twenty microliter of a 758 μM solution of oligomer **4** was added to 80 μL of fresh human serum. A 10-μL sample was removed immediately and the remainder incubated at 37 °C. Aliquots (10 μL) were taken at 0, 1, 2, 3, 4, 6 and 24 h, and quenched with 10 μL of 7 M urea solution at a temperature of 95 °C for 2 min. Stability was analysed by RP-HPLC by using a Phenomenex Juppiter C18 300 Å (5 μm, 4.6 × 250 mm) column with a flow rate of 0.8 ml/min by using a linear gradient of 10 % (for 5 min) to 45 % B in A over 20 min.

Computational methods

The molecular model (Fig. 2b) of the interaction between the cationic nucleopeptide and nucleic acid was realized by WebLab Viewer Lite 4.2 software (Molecular Simulations, Inc., San Diego-CA, 2000).

Results and discussion

The synthesis of the new diaminopropanoic acid-based thymine monomer (**2**), suitably protected for peptide solid phase synthesis (Fmoc chemistry), is reported in Scheme 1 and starts from the commercial Boc/Fmoc-protected 2,3-diaminopropanoic acid **1**. After removal of the Boc group, achieved by acid treatment, *Nβ*-Fmoc-L-2,3-diaminopropanoic acid **2** was obtained in high yield. Subsequently, compound **2** was reacted with thyminyl acetic acid by using HATU/DIEA as a coupling system. After solvent removal, precipitation from water and filtration Fmoc-protected nucleoaminoacid **3** was obtained in 89 % yield. LC-ESIMS (Fig. S1 and S2) characterization confirmed the identity of both compound **2** and monomer **3**.

Subsequently, a hexathymine nucleopeptide (**4**) was synthesized in solid phase by using the monomer **3** and the commercial Fmoc-L-Lys(Boc)-OH. The introduction of the nucleoamino acid residues was performed by the strategy of Sforza et al. (2002) which minimizes racemization during the coupling steps, employing HATU/TMP as a coupling system without preactivation (Scheme 2). A glycine residue was introduced at N-terminus of the nucleopeptide in order to avoid side reactions due to the nucleophilicity of the beta-amino group of the final diaminopropanoic unit.

After the chemical synthesis, the oligomer was cleaved from the solid support by acidic treatment (TFA/TIS/H_2O, 95:2.5:2.5, v/v/v) and purified by RP-HPLC on a C-18 column with a linear gradient of CH_3CN (0.1 % TFA) in H_2O (0.1 % TFA).

LC-ESIMS characterization (positive ions) confirmed the identity of the nucleopeptide (Fig. S3) which was obtained in 27 % overall yield. The compound resulted well soluble in water and did not show any tendency to aggregate.

Furthermore, the structural characteristics of the diaminopropanoic acid-based nucleopeptide, as well as its ability to interact with complementary DNA and RNA, were

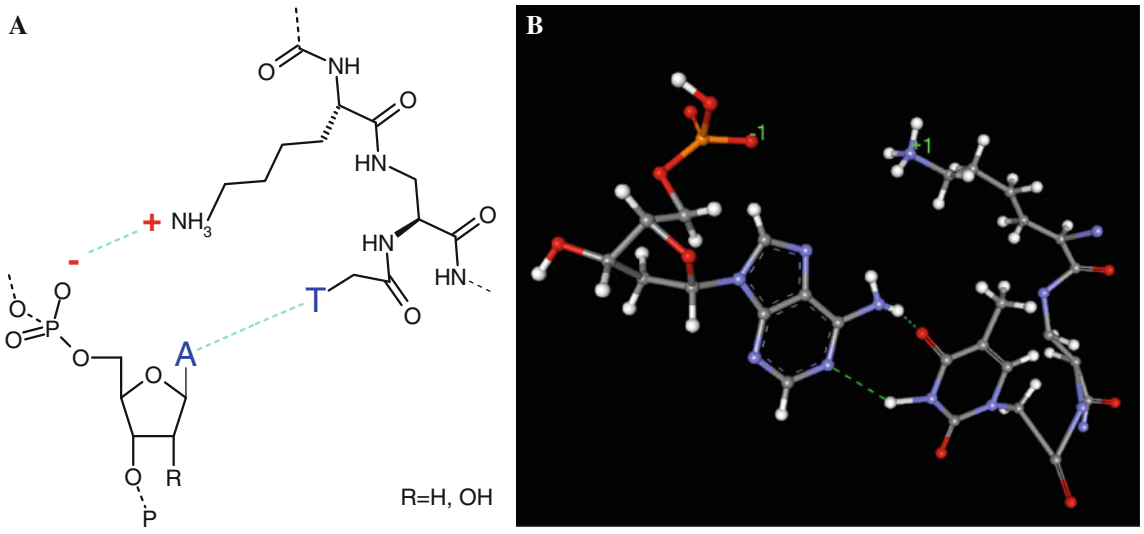


Fig. 2 Schematic representation (**a**) and molecular model (**b**) of the interaction occurring between the cationic nucleopeptide and a nucleic acid as hypothesized in the design of the analog

investigated by CD spectroscopy. First, the CD profile of the single strand in 10 mM phosphate buffer (pH 7.5) was analysed in order to evaluate any pre-organization of the molecule. By examining the CD behaviour of the molecule **4** in the range 190–320 nm at 10 °C, no significant α-helical contribution was detected for the single strand. However a weak band at 272 nm (Fig. 3) was observed according to other reports on thymine containing chiral nucleopeptides (Roviello et al. 2010a).

CD binding experiments on the hexathymine nucleopeptide with complementary DNA and RNA (polyA) were then performed in a tandem cell recording the sum CD spectrum of the separated components and the mix CD spectrum, recorded after cell mixing, in order to evaluate its ability to interact with DNA and RNA, an important property in view of possible biomedical applications of the new molecule.

More in detail, 48 nmol in nucleobase (T) of nucleopeptide **4** in 10 mM phosphate buffer pH = 7.5 were introduced in one of the two tandem cell reservoirs. Subsequently, a solution of dA_{12} DNA or polyA RNA in 10 mM phosphate buffer pH = 7.5 was introduced in the other reservoir and "sum" CD spectra were recorded in both cases. The CD due to the nucleopeptide single strand resulted weak as compared to the strong bands of the DNA and RNA evident in the "sum" CD spectrum.

After mixing the two solutions, a certain difference in the shape and intensity of the CD profiles observed between the sum and mix spectra revealed an interaction between the nucleopeptide and both nucleic acids. Furthermore, a more evident variation in CD signal was observed in both cases when a further aliquot of **4** was added to the complex solution (red line, Figs. 4, 5). In particular, the pronounced difference in CD spectra in case of the complex obtained with RNA suggests a significant conformational variation of RNA induced by interaction with the cationic nucleopeptide.

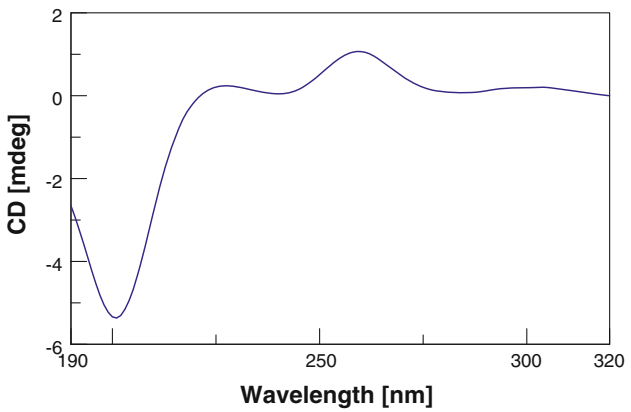


Fig. 3 CD profile relative to a 8 μM solution of the nucleopeptide **4** (in 10 mM phosphate buffer, pH 7.5) at 10 °C

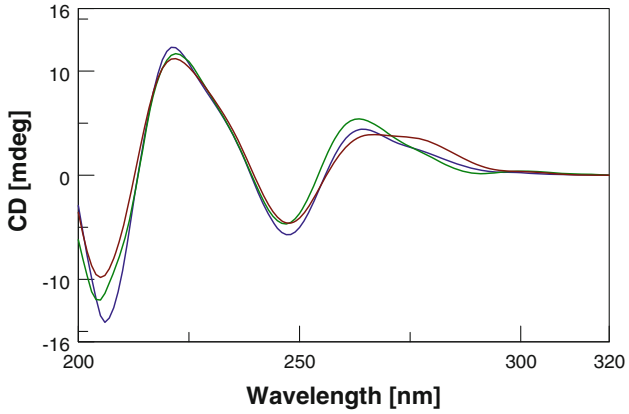


Fig. 4 CD binding study: 48 nmol in T of nucleopeptide **4** + 48 nmol in A of DNA (dA_{12}) before (*green*) and after (*blue*) mixing; 96 nmol in T of nucleopeptide **4** + 48 nmol in A of DNA (*red line*). All spectra were recorded at 10 °C in 10 mM phosphate buffer pH = 7.5 (colour figure online)

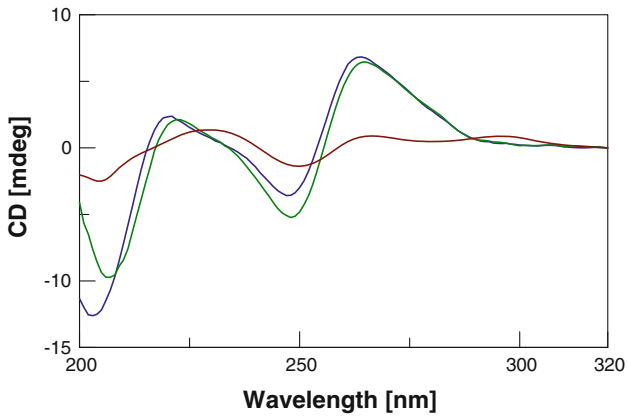


Fig. 5 CD binding study: 48 nmol in T of nucleopeptide **4** + 14 nmol in A of RNA (polyA) before (*blue*) and after (*green*) mixing; 96 nmol in T of nucleopeptide **4** + 14 nmol in A of RNA (*red line*). All spectra were recorded at 10 °C in 10 mM phosphate buffer pH = 7.5 (colour figure online)

Serum stability assay

Finally, the enzymatic resistance of the nucleopeptide was studied by incubating **4** in 80 % fresh human serum (the concentration of **4** was 152 μM) at 37 °C and analyzing by RP-HPLC samples withdrawn from the reaction mixture at various times (0, 1, 2, 3, 4, 5, 6 and 24 h).

By analyzing the results of the stability assay (Fig. 6), it can be deduced that the cationic nucleopeptide is stable in human serum at 37 °C for up to 24 h.

Conclusions

In this work we reported the synthesis, purification and characterization of a novel Fmoc-protected nucleoamino-acid based on L-diaminopropanoic acid, suitable for the

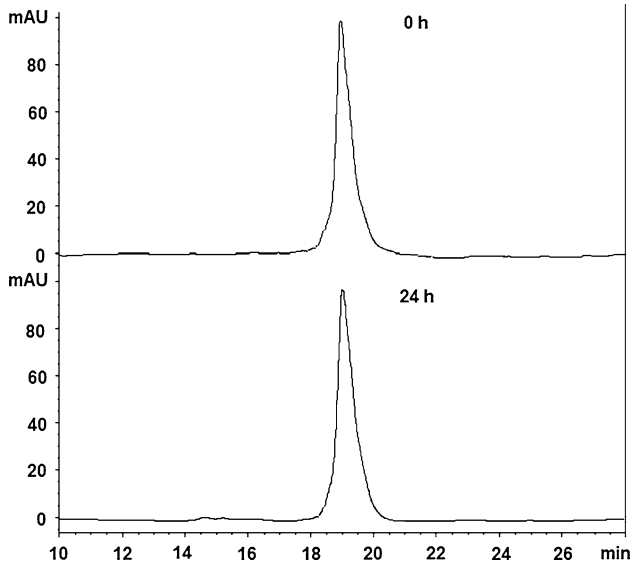


Fig. 6 Serum stability HPLC profiles at 0 and 24 h (experiments performed on nucleopeptide **4** in 80 % fresh human serum)

solid phase synthesis of nucleopeptides, and its oligomerization to the sequential oligomer **4** with six thymine monomers and six L-lysine units, by making use of a solid phase synthetic route. After the chemical synthesis, the cationic nucleopeptide was purified by HPLC and characterized by LC-ESIMS (positive ions) which confirmed the identity of the desired product. Binding experiments, performed by CD spectroscopy, showed that this peptide-like analog of DNA, which resulted well soluble in water and did not present any tendency to self-aggregate, was able to interact with complementary DNA and RNA molecule inducing significant conformational variations of the structure of the nucleic acid, as observed in case of the experiment with RNA. Finally, enzymatic resistance of the oligomer **4** was proven by human serum stability assays. Taken together, all these findings encourage to deepen the study of the properties of the cationic nucleopeptide herein described as a novel tool able to interact with DNA and RNA which could be beneficial in the medical research.

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Conflict of interest The authors state that there is no conflict of interests.

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