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ORIGINAL ARTICLE

Synthesis of a thymine-functionalized nucleoamino acid for the solid phase assembly of cationic nucleopeptides

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Abstract In this work, we report the synthesis of a thymine-functionalized nucleoamino acid suitable for the solid phase synthesis of nucleopeptides. The monomer was obtained in solution starting from commercial compounds and after NMR (^{1}H and ^{13}C) and ESIMS (positive ions) characterization it was used for the assembly of a cationic nucleopeptide obtained by sequentially introducing underivatized L-lysine units and nucleoamino acid monomers. After detachment from the resin, performed in acidic conditions, the oligomer was purified by HPLC and characterized by LC-ESIMS (positive ions) which confirmed the identity of the thymine-based nucleopeptide. The cationic nucleobase-containing peptide, well soluble in water, was studied by CD spectroscopy which allowed us to exclude any helical pre-organization of the nucleopeptide in the experimental conditions used. Furthermore, CD behavior of the oligomer at different temperatures was also studied as described in this work.

Keywords Nucleopeptide · L-Lysine · CD

Abbreviations

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
Lys	L-Lysine
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

Introduction

In several research studies, it has been shown that various modifications of sugar phosphodiester linkage of nucleic acids can lead to oligonucleotide analogs with useful properties (Abramova et al. 1991; Bell and Micklefield 2009; D'Alonzo et al. 2011; D'Onofrio et al. 2005; Zarra et al. 2009; Zatsepin et al. 2003). Interestingly, an extensive research work was conducted by many researchers on peptide-based analogs of nucleic acids (also referred to as nucleopeptides) which in some cases were characterized by a good intrinsic cell-membrane permeability as well as a significant ability to interact with natural nucleic acids leading to complexes of high thermal stability (Roviello et al. 2010a). Some

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nucleopeptides were found to be able to form supramolecular structures or bind heteroaromatic molecules (Petraccone et al. 2005; Roviello et al. 2011, 2012; Simeone et al. 2012). Remarkable cell permeability and specific and stable DNA binding were found in case of positively charged analogs with peptide-like backbones (Katritzky and Narindoshvili 2008; Dragulescu-Andrasi et al. 2006; Calabretta et al. 2009). Several attempts to use real peptides as alternative oligonucleotide linkages have also been reported, leading to chiral nucleopeptides with useful characteristics (van der Laan et al. 1998; Roviello et al. 2009). Furthermore, examples of peptide-like analogs of DNA with long side chains able to form stable complexes with natural oligonucleotides of high sequence specificities were also reported in literature (Wada et al. 2000; Sawa et al. 2010).

Natural diamino acids are frequently used as building blocks for the construction of artificial analogs of nucleic acids since they offer the possibility to functionalize the main peptide backbone, obtained by the oligomerization of the diamino acid moieties also in combination with other amino acids, by nucleobases introduced through amidation to one of the two amino groups of the diamino acid residues (Roviello et al. 2006, 2007, 2010b).

Taking into consideration the above findings, as well as the useful characteristics of diamino acid-containing nucleopeptides, we designed and realized an oligonucleotide analog, characterized by an oligolysine backbone comprising both nucleobase-functionalized and underivatized L-lysine moieties in an alternating sequence. In our design, the diamino acid moieties allow for the anchorage to the DNA nucleobases by means of an amide bond to the epsilon amino moiety, whereas the unfunctionalized L-lysine residues confer a positive charge to the oligonucleotide analog, characteristic of potential significance for improving the water solubility as well as the ability to recognize the negatively charged DNA and RNA targets.

Materials and methods

Chemicals

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

Apparatus

^1H NMR and ^{13}C NMR spectra were recorded at 25 °C on Varian unity 600 MHz spectrometers. Chemical shifts (δ) are given in parts per million (ppm) and all coupling constants (J) in hertz. 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 analyzed by LC-ESIMS, 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, 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^{-1}.

Semi-preparative purifications were performed by RP-HPLC on a Hewlett Packard/Agilent 1100 series, equipped with a diode array detector, 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^{-1}. Samples containing nucleopeptides (crude or purified) were lyophilized in an 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 quartz cell with a light path of 1 cm.

Synthesis of the Fmoc-protected thymine-bearing diamino acid (**3**): *N*α-Fmoc-L-2,6-diaminohexanoic acid, **2** (Scheme 1)

Commercial Boc/Fmoc-protected 2,6-diaminohexanoic **1** (Fmoc-L-Lys(Boc)-OH, 230 mg, 0.49 mmol) was treated with 1:1 TFA/DCM solution (10 ml) at 45 °C under stirring. After 1.5 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 dried under vacuum. The identity of the desired intermediate product **2**, obtained in 97 % yield (175 mg, 0.47 mmol), was confirmed by LC-ESIMS. The compound was ≥95 % pure by

HPLC analysis. ESIMS (Figure S1) *m/z* 370.76 (found), 369.45 (expected for $[C_{21}H_{24}N_2O_4+H]^+$); 737.89 (found), 737.89 (expected for $[2x(C_{21}H_{24}N_2O_4)+H]^+$).

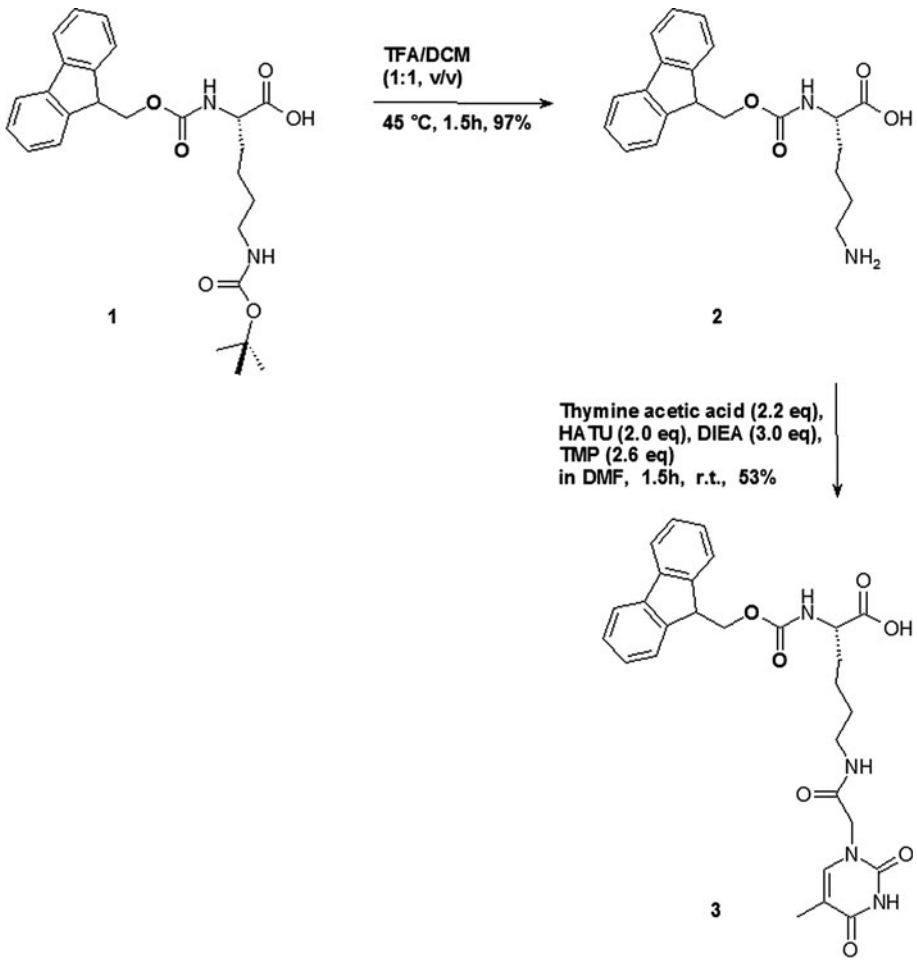
*N*α-Fmoc-*N*ε-(thymin-1-ylacetyl)-L-2,6-hexanoic acid, **3** (Scheme 1)

The obtained Fmoc-protected diamino hexanoic acid **2** (1 eq, 175 mg, 0.47 mmol) was dissolved in anhydrous DMF (2 ml), treated with DIEA (0.9 eq, 83 µl, 0.42 mmol) and TMP (0.6 eq, 42 µl, 0.28 mmol), and reacted with TCH_2COOH (2.2 eq, 200 mg, 1.03 mmol) which was previously preactivated by HATU (2.0 eq, 372 mg, 0.94 mmol) and DIEA (2 eq, 175 µl, 0.94 mmol)/TMP (2 eq, 140 µl, 0.94 mmol) in DMF (2 ml) for 2 min. After 1.5 h, the solvent was removed under vacuum and the crude was suspended in water (50 ml) aided by sonication. The resulting precipitate was collected by filtration and the obtained product was identified by NMR and MS spectrometry as the desired product **3** (133 mg, 0.25 mmol, 53 % yield). The compound was ≥95 % pure by HPLC analysis. LC-ESIMS (Figure S2) *m/z* 536.90 (found), 535.58 (expected for $[C_{28}H_{30}N_4O_7+H]^+$); *m/z* 558.35 (found), 557.56 (expected for $[C_{28}H_{30}N_4O_7+Na]^+$); *m/z* 574.42 (found), 573.68 (expected for $[C_{28}H_{30}N_4O_7 + K]^+$); δ_H (400 MHz, DMSO-d6) 11.31 (1H, s, NH thymine), 8.34 (1H, t, $J = 5.6$, CH_2NH amide), 7.94 (2H, d, $J = 7.6$, aromatic CH Fmoc), 7.78 (2H, d, $J = 7.2$, aromatic CH Fmoc), 7.67 (1H, d, $J = 8.0$, $CH_\alpha NH$ amide), 7.48–7.36 (5H, m, aromatic CH Fmoc, CH thymine), 4.34–4.26 (5H, m, CH_2 acetyl linker, FmocCH-CH2), 3.96 (1H, m, CH_α), 3.11 (2H, m, CH_2NH), 1.79 (3H, s, CH_3 thymine), 1.78–1.33 (6H, m, CH_2CH_α, $CH_2CH_2CH_\alpha$, CH_2CH_2NH); δ_C (100 MHz, DMSO-d6) 173.9, 166.6, 164.4, 156.2, 151.0, 143.8, 142.4, 140.7, 127.6, 127.1, 125.3, 120.1, 107.9, 65.6, 53.7, 49.3, 46.6, 38.5, 30.4, 28.5, 23.0, 11.9.

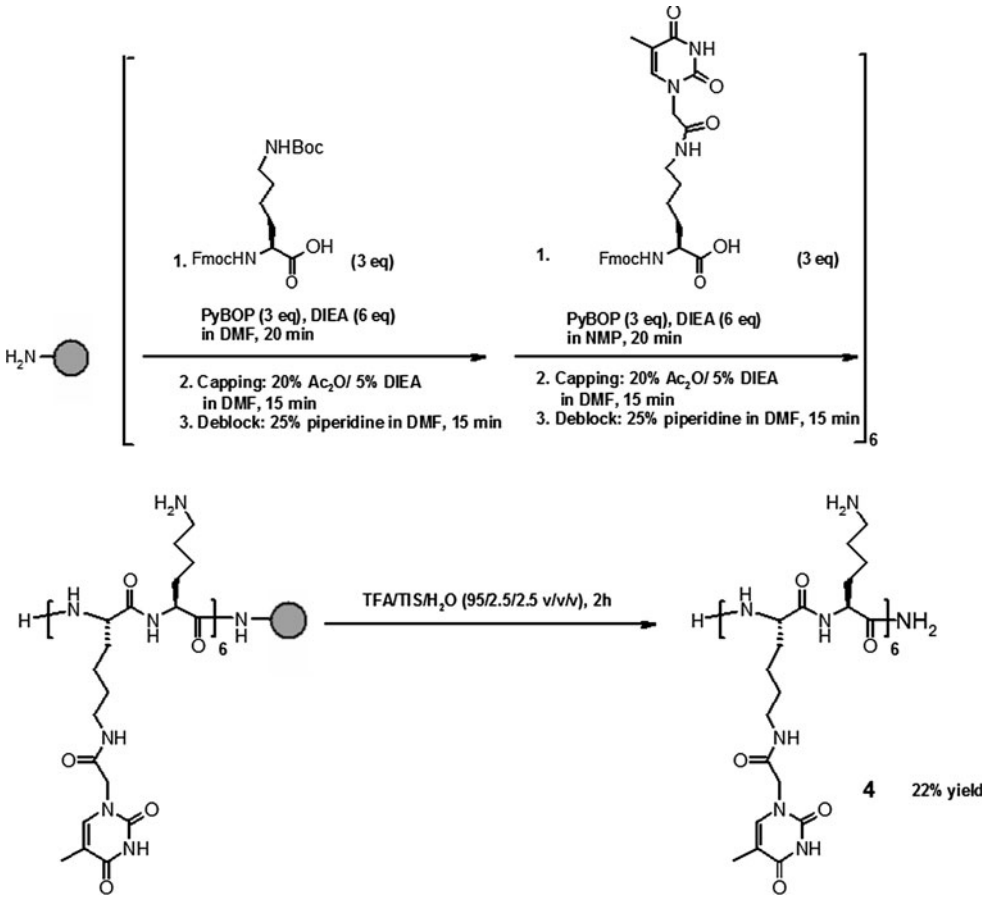
Solid phase synthesis of oligomer **4**

Oligomer **4** was assembled on Rink-amide MBHA resin (0.50 mmol g^{-1}, 8.0 mg) using the synthetic strategy described in Scheme 2. More particularly, after Fmoc removal from the resin (by treatment with 25 % piperidine

Scheme 1 Synthesis of monomer **3**



Scheme 2 Synthesis of oligomer **4**



in DMF for 30 min), a mixture of Fmoc-Lys(Boc)-OH or monomer **3** (120 µL of 0.1 M solution in DMF, 12 µmol, 3 eq), PyBOP (76 µl of 0.16 M solution in NMP, 12 µmol, 3 eq) and DIEA (4 µL, 24 µmol, 6 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 DMF, and, subsequently, unreacted amino groups were capped by treatment with Ac_2O (20 %)/DIEA (5 %) in DMF for 15 min. Fmoc group was removed by treatment with 25 % piperidine in DMF for 15 min. The above-described procedure for the sequential introduction of the underivatized lysines and nucleoamino acid residues was repeated 12 times. A 22 % overall yield was estimated by Fmoc UV test. The cleavage from the resin and deprotection of the nucleopeptide were performed by treatment with a di TFA/TIS/H_2O (95 %/2.5 %/2.5 %) solution for 90 min. After precipitation with cold diethyl ether, the sample was dissolved 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: $t_R = 19.5$ min. The purified product was dissolved 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 (51,600 M^{-1} cm^{-1}) was calculated by the molar extinction coefficient of thymine PNA monomer (8,600 M^{-1} cm^{-1}). UV quantification of the purified product gave 0.3 µmol of product **4** (8 % yield); the compound was ≥95 % pure by HPLC analysis. ESIMS (Figure S3) *m/z* 639.85 (found), 639.00 (expected for $[C_{114}H_{183}N_{37}O_{30}+4H]^{4+}$); 853.52 (found), 851.66 (expected for $[C_{114}H_{183}N_{37}O_{30}+3H]^{3+}$); 1,277.90 (found), 1,276.99 (expected for $[C_{114}H_{183}N_{37}O_{30}+2H]^{2+}$).

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 Hellma quartz cells. CD spectra were obtained using the following parameters: scan speed 50 nm min^{-1}, data pitch 2 nm, band width 2 nm, response 4 s, and 5 accumulations.

Results and discussion

The synthesis of the diaminohexanoic acid-based thymine monomer (**3**), suitably protected for peptide solid phase synthesis (Fmoc chemistry), is reported in Scheme 1 and starts from the commercial Boc/Fmoc-protected 2,3-

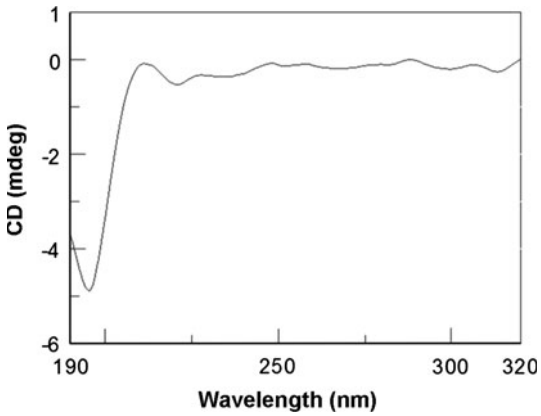


Fig. 1 CD profile relative to 2.5 μM solution of nucleopeptide **4** (in 10 mM phosphate buffer, pH 7.5) at 15 °C

diaminohexanoic acid **1**. After removal of the Boc group, achieved by acid treatment, *N*α-Fmoc-L-2,6-diaminohexanoic acid **2** was obtained in quantitative yield. Subsequently, compound **2** was reacted with thyminyl acetic acid using HATU/DIEA as a coupling system. After solvent removal, precipitation from water and filtration, Fmoc-protected nucleoaminoacid **3** was obtained in 53 % yield. LC-ESIMS (Figure S1 and S2) characterization confirmed the identity of both compound **2** and monomer **3**.

Subsequently, a hexathymine nucleopeptide (**4**) was synthesized in solid phase using the monomer **3** and the commercial Fmoc-L-Lys(Boc)-OH with PyBOP/DIEA in NMP as a coupling system (Scheme 2). After 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 (Figure S3) which was obtained in 22 % overall yield. The compound was well soluble in water and did not show any tendency to aggregate.

CD conformational studies

Furthermore, the structural characteristics of the diaminohexanoic acid-based nucleopeptide were investigated by CD spectroscopy. First, the CD profile of the single strand in 10 mM phosphate buffer pH 7.5 was analyzed to evaluate any pre-organization of the molecule. By examining the CD behavior of the molecule **3** in the range 190–320 nm at 15 °C (Fig. 1), no significant α-helical contribution was detected for the single strand.

Subsequently, CD behavior under temperature variation was evaluated, and more particularly, CD spectra for 2 μM solution of nucleopeptide **4** in 5 mM phosphate buffer (pH 7.5) were recorded in the 198–320 nm range at several

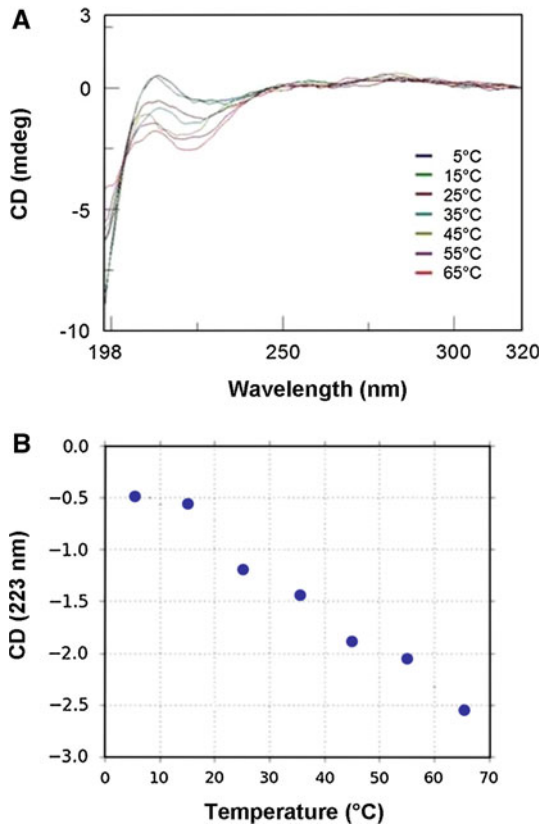


Fig. 2 Overlapped CD spectra at various temperatures (**a**) and variation of CD value registered at 223 nm as a function of temperature (**b**), relative to 2 μM solution of **4** in 5 mM phosphate buffer (pH 7.5)

temperatures. Based on this study, a variation of CD spectrum as a consequence of temperature increase was observed (Fig. 2), and more particularly, a decrease of the CD signal around 223 nm was observed with increasing temperature from 5 to 65 °C. This behavior can be due to small intramolecular conformational changes of nucleopeptide or to the perturbation of a supramolecular assembly based on nucleopeptide units whose structure undergoes changes as a consequence of temperature increase, the latter being a hypothesis to be confirmed by further investigation.

Conclusions

In conclusion, in this study we have described the synthesis and characterization of a thymine-based nucleoamino acid and its oligomerization to a thymine-functionalized oligolysine containing six thyminyl lysines and six unfunctionalized L-lysine units. This cationic nucleopeptide was well soluble in water and did not present any tendency to self-aggregate. Moreover, CD experiments suggested that the thyminyl oligolysine does not undergo any helical pre-

organization under the experimental conditions explored by us. The CD behavior observed for oligomer **4** under temperature variation can be due to small intramolecular conformational changes or the perturbation of supramolecular networks based on oligomer **4**, a hypothesis that, together with other biological aspects such as the nucleic acid-binding ability of the nucleopeptide, needs further investigation.

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

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