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PAPER

Evidences for supramolecular organization of nucleopeptides: synthesis, spectroscopic and biological studies of a novel dithymine L-serine tetrapeptide†

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This work concerns a dithymine tetrapeptide, which can be seen as a new analogue of a dinucleoside monophosphate, made of both unfunctionalized and thymine-containing L-serine units alternated in the sequence. The new nucleopeptide was obtained on the solid phase by two different synthetic strategies. The first one is suitable to easily realize nucleopeptides with homonucleobase sequences, obtained by assembling an oligoserine backbone and then simultaneously coupling the free serine hydroxyl groups with the carboxymethylated nucleobase. The other strategy, which makes use of a Fmoc-protected nucleo-L-serine monomer, allows for the obtainment of nucleopeptides with mixed nucleobase sequences. CD spectroscopic studies and laser light scattering experiments, performed on solutions of the novel nucleopeptide, suggested the formation of supramolecular networks based on the self-assembly of the dithymine tetrapeptide molecules. Furthermore, CD binding studies with natural nucleic acids revealed a very weak interaction between the nucleopeptide and DNA (but not RNA). Molecular networks based on this biodegradable and water-soluble nucleopeptide, which is more resistant in plasma than standard tetrapeptides (and oligopeptides), contain a hydrophobic core which could provide the necessary environment to incorporate poorly water-soluble drugs, as evidenced by fluorescence spectroscopy. Furthermore, our studies evidenced that the structure of the tetrapeptide-based supramolecular assembly can be modified by metal ions as evidenced by UV interaction studies with Cu^{2+}.

Introduction

Many investigations regarding novel antigene or antisense strategies have shown that the ribose phosphodiester linkage can be replaced in novel oligonucleotide mimics by several modifications.[1] For example, several attempts to use a polyamide backbone as an alternative oligonucleotide linkage have been reported, allowing for the obtainment of several DNA analogues with interesting properties.[2–9] As a general rule, in a nucleopeptide backbone two α-aminoacid units are isomorphous in terms of their length (six bonds) to one sugar-phosphate residue of the DNA and RNA backbone. Therefore, the repeating unit of a potential nucleic acid-binding nucleopeptide could be built of two amino acid moieties, one of which derivatized with the nucleobase, directly joined to one another by a peptide bond.[10]

In the last decades, a great relevance has been attributed to hydrogels as well as water-soluble macromolecular networks useful for the incorporation and the release of genes and drugs.[11] These polymeric systems are governed by non-covalent bonds occurring between the subunits and can be based on one or more types of monomeric units. Among the different classes of molecules able to form supramolecular architectures it is worth to mention systems characterized by polynucleobase-molecules such as nucleic acids, which are particularly important in nanomedicine,[12] and peptide nucleic acids,[13] as well as systems characterized by monomeric units bringing a single nucleobase which can form gels based on the cooperative effects of the complementary nucleobases.[14] Taking into account all these considerations we designed and realized a novel analogue (**1**) of TpT DNA dinucleoside monophosphate (**2**, Fig. S1, ESI†), characterized by a peptide backbone comprising both nucleobase-functionalized and free L-serine moieties alternated in the sequence. In this oligomer the atoms of the backbone which bring the DNA bases are separated by the same number of bonds (*i.e.* 6, Fig. S1, ESI†) found in DNA, and the nucleobases are anchored to the hydroxyl groups of the L-serine units by means of ester bonds.

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Underivatized L-serines were introduced as spacers between the nucleobase-carrying aminoacids in order to achieve the proper distance between two consecutive nucleobases, as well as to improve the water-solubility of the resulting nucleopeptide, as expected for the presence of the hydrophilic hydroxyl moieties. This TpT DNA dinucleoside monophosphate analogue is expected to be more resistant to enzymatic degradation than DNA but also biodegradable due to its real peptide nature. The self-assembly properties as well as the ability to interact with nucleic acids of the novel alternate L-serine-based nucleopeptide were investigated, as described in the present work.

Results and discussion

Solid phase synthesis of homonucleobase–oligoserines

We realized an easy synthetic route to homonucleobase–oligoserines (Fig. 1). Briefly, a tetrapeptide was assembled in the solid phase by using both commercial Fmoc-L-Ser(tBu)-OH and Fmoc-L-Ser-OH (the latter was obtained as described below in this work), and PyBOP/DIPEA as the activating system. By previous experiments we did not find any reactivity of the free hydroxyl groups under the coupling conditions used (data not shown). No capping step was performed to avoid the acetylation of the free hydroxyl groups, which were essential for the subsequent functionalization with the nucleobases. Then, the free hydroxyl groups were esterified with the nucleobase acetic acid (TCH_2COOH) by using DCC/DMAP as the activating system. After final Fmoc removal a UV Fmoc test revealed a 67% overall yield for the tetraserine backbone synthesis.

Subsequently, the crude oligomer was detached from the resin by acidic treatment (95% TFA), precipitated with cold diethyl ether and purified by RP-HPLC on a C_{18} column. ESI-MS (positive ions) confirmed the identity of the dinucleotide analogue (Fig. S2, ESI†).

Synthesis of the Fmoc-protected monomer 5 for the solid phase assembly of nucleopeptide 1

We realized a convenient and fast synthetic route to chiral Fmoc nucleo-L-serine monomer, in which the (*S*)-2-amino-3-hydroxypropanoic moiety is connected to the DNA nucleobase by an ester bond, suitable for the solid phase synthesis of nucleopeptides. The nucleobase-containing monomer was synthesized starting from the commercially available Fmoc-L-Ser(tBu)-OH (**3**, Fig. 2). In the first synthetic step the *tert*-butyl group was selectively removed with trifluoroacetic acid to give the free hydroxyl group in β position (compound **4**, Fig. 2). ESI-MS (positive ions) confirmed the identity of this intermediate (Fig. S3, ESI†).

Subsequently, Fmoc-L-Ser-OH **4** was coupled with the nucleobase acetic acid under different synthetic conditions with the best results coming from the use of DIPC/DMAP in DMF as solvent (Fig. 2). After RP-HPLC purification the desired product **5** was obtained in 30% yield and characterized by $^{13}C/^{1}H$ NMR and ESIMS (Fig. S4, ESI†). Subsequently, the nucleo-tetrapeptide **1** was assembled in the solid phase using an alternative synthetic strategy employing the coupling of both monomer **5** and the commercial Fmoc-L-Ser(tBu)-OH achieved with PyBop/DIPEA as the activating system (Fig. 3). The yield for the incorporation of the novel nucleoaminoacid **5** was about 80%, while the overall yield for the obtainment of **1**, calculated on the basis of the UV Fmoc test, was about 50%. ESI-MS (positive ions) confirmed the identity of the oligomer.

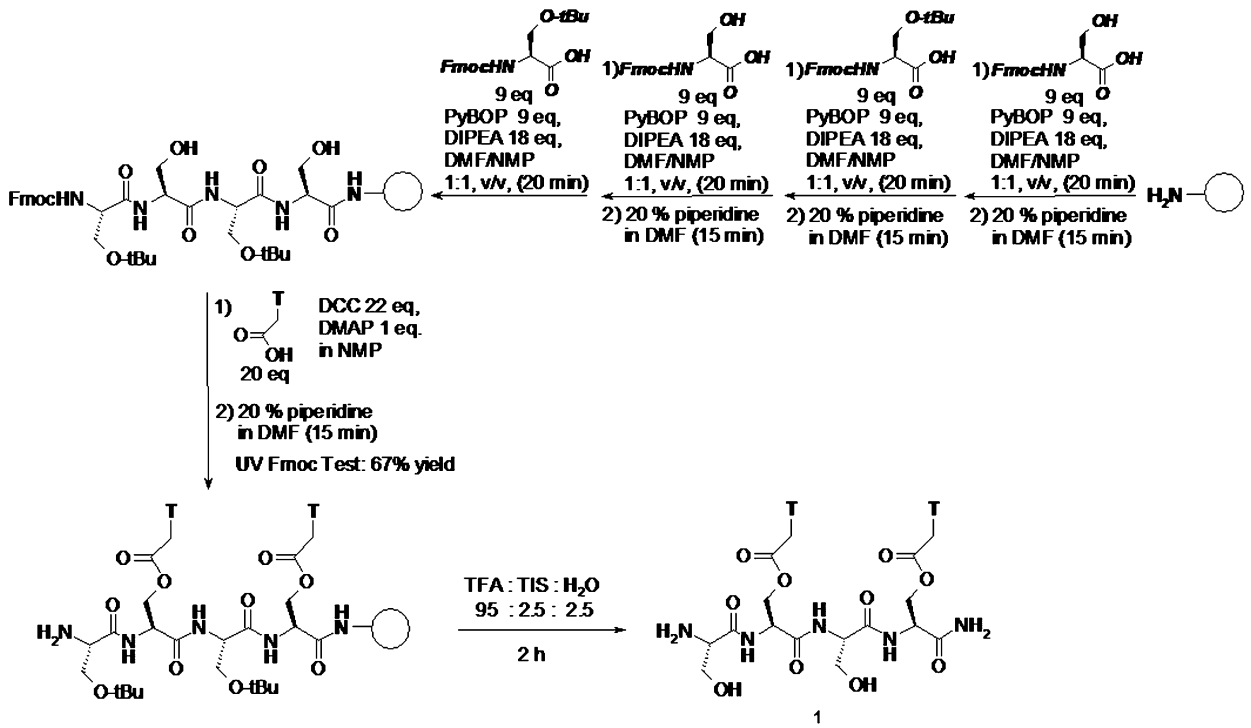

Fig. 1 Synthesis of the L-serine-based nucleo-tetrapeptide.

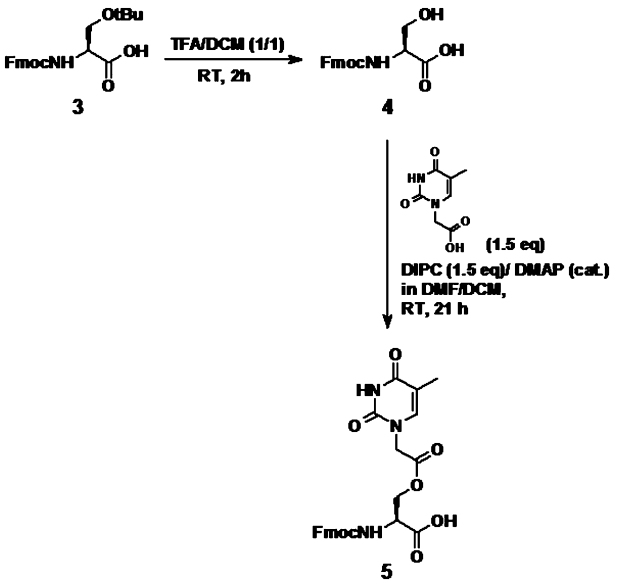

Fig. 2 Synthesis of monomer **5**.

Interestingly, the route to the nucleopeptide **1** which makes use of monomer **5** provided an overall yield higher than that achieved by the strategy involving the esterification on the solid phase with the nucleobase–acetic acid (Fig. 2). However, the latter approach minimizes the number of synthetic and purification steps required and results, thus convenient for the synthesis of homonucleobase–oligoserines. On the other hand, the strategy described in Fig. 3, which can be extended also to the other nucleobases (*i.e.* A, G and C) by using the appropriate nucleobase–acetic acids, allows for the synthesis of nucleopeptides with mixed sequence.

Supramolecular self-assembly of nucleopeptides—CD spectroscopy

Molecular self-assembly of **1** was investigated using CD by monitoring the value of a CD signal at 200 nm at different nucleopeptide concentrations (Fig. 4a). An intense negative peak at 200 nm appeared upon increasing the concentration of nucleopeptide from 10 to 78 μM. By plotting the CD signal at 200 nm as a function of the nucleopeptide concentration, we observed two main slope changes (Fig. 4b), corresponding probably to two tridimensional states of the supramolecular network. The CD spectra of the peptide did not present any significant change other than a decrease in molar ellipticity indicating that there was no substantial variation in the nucleopeptide conformation. Hence, it can be concluded that the conformational preference of dithyminyl tetraserine is not changed during the self-assembly.

By examining the CD spectra at higher concentration levels (275–550 μM), it was possible to appreciate not only a further reduction in CD intensity recorded at 200 nm (Fig. 4d), but also an induced CD contribution of the nucleobases consisting of a positive band at about 270 nm and a negative band at about 295 nm (Fig. 4c).

The CD spectra relative to two nucleopeptide solutions at different values of pH and temperatures were also recorded (Fig. 5). A major degree of supramolecular organization of the dithymine nucleopeptide was detected at pH 7.5 and at temperatures lower than 30 °C.

Static laser light scattering

Static laser light scattering experiments were performed on nucleopeptide solutions at different concentrations. The data collected were in accord with the formation of supramolecular networks whose size did not vary significantly with the

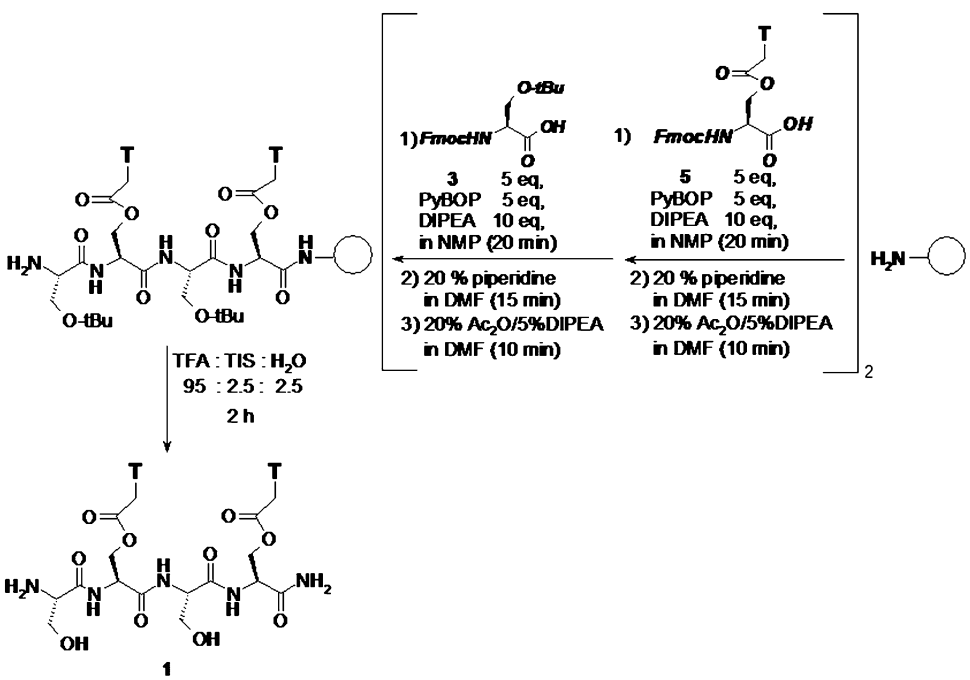

Fig. 3 Synthesis of nucleo-tetrapeptide **1** by using monomer **5**.

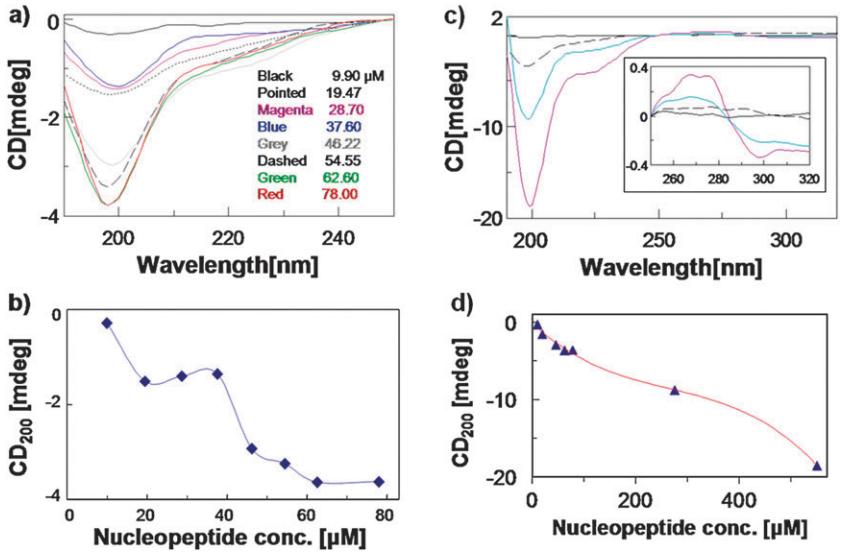

Fig. 4 (a) CD spectra of nucleopeptide **1** in phosphate buffer (pH = 7.5) at various concentrations; (b) CD value at 200 nm as a function of the nucleopeptide **1** concentration (10–78 μM); (c) CD spectra of nucleopeptide **1** in phosphate buffer (pH = 7.5): 10 (black line); 54 (dashed black), 275 (cyan) and 550 μM (purple); (d) CD values at 220 nm as a function of the nucleopeptide **1** concentration (10, 19, 46, 63, 78, 275 and 550 μM).

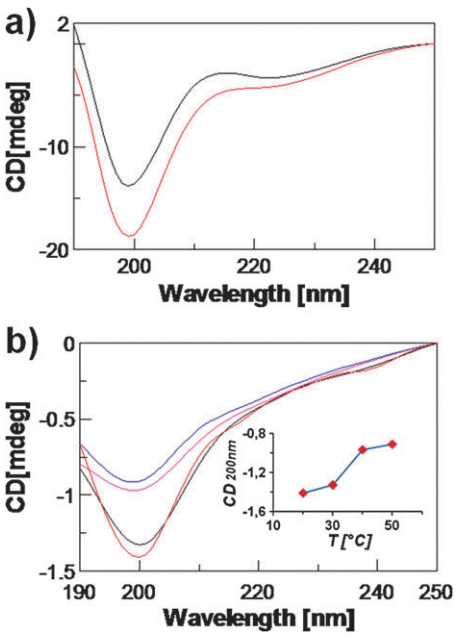

Fig. 5 CD spectra of nucleopeptide **1** (a) 550 μM at pH 6 (black) and pH 7.5 (red); (b) 30 μM at pH 7.5 at 20 (red), 30 (black), 40 (pink) and 50 (blue) °C.

concentration in the 25–280 μM range (with a mean hydrodynamic radius of about 45 nm estimated in this range) but significantly increased after the 300 μM concentration level (Fig. 6).

Fluorescence spectroscopy

Fluorescence aggregation studies on **1** were also carried out using 8-anilino-naphthalene sulfonate (ANS) as an external probe. Indeed, ANS can be used as a sensitive probe to monitor the assembly of oligopeptides. Representative fluorescence spectra of ANS in phosphate buffer in the absence

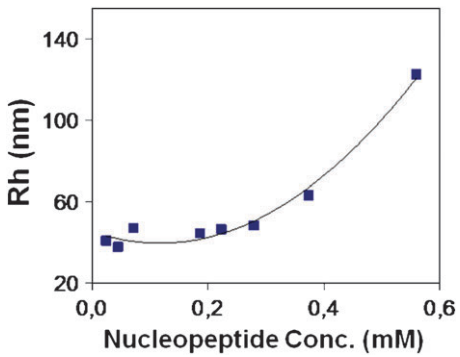

Fig. 6 Hydrodynamic radius *vs.* nucleopeptide concentration.

and presence of various concentrations of nucleopeptide **1** are shown in Fig. 7a. For each concentration value, ANS fluorescence stabilized after about 90 min. The changes in emission were tracked until the system reached equilibrium. Fluorescence intensity of ANS at 530 nm was plotted against peptide **1** concentrations (inset of Fig. 7a). The intensity increased with increasing the peptide concentration, indicating that the ANS binding site became more nonpolar on aggregation. From the plot of ANS fluorescence intensity *vs.* nucleopeptide concentration (Fig. 7a, inset), a change in the slope at about 60 μM concentration can be detected, corresponding to the critical aggregate concentration of the nucleopeptide. Furthermore, we evaluated the ability of nucleopeptide **1** to form supramolecular networks also by kinetic FITC fluorescence experiments. We observed a time-dependent effect of the nucleopeptide on FITC fluorescence (Fig. 7b). In particular, the increase of FITC fluorescence intensity in time after addition of **1** sustains the hypothesis of molecular networking and the formation of a hydrophobic core which provides the necessary environment to incorporate poorly water-soluble molecules, such as FITC (see Fig. 7b). The changes in emission were tracked until the system reached equilibrium (2 h).

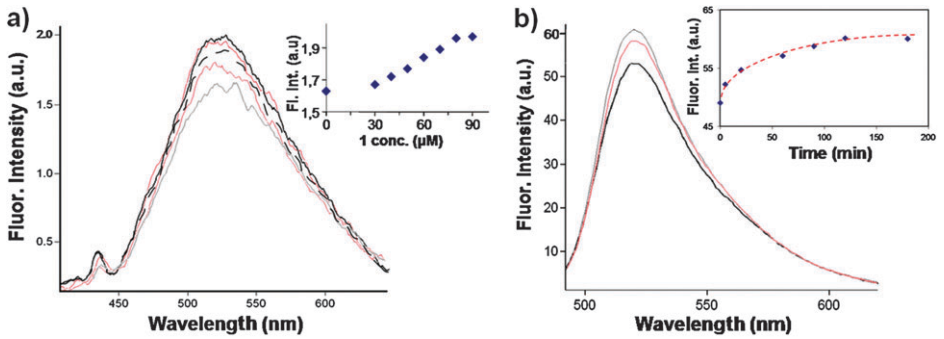

Fig. 7 (a) Fluorescence titration of ANS (30 μM) with nucleopeptide **1** (0–90 μM) in phosphate buffer (pH = 7.5). ANS alone (grey line); 50 (magenta), 70 (dashed black), 80 (red) and 90 μM (black) of **1** added. Fluorescence spectra relative to the 30, 40 and 60 μM concentration of **1** were omitted for clarity; inset of Fig. 7a: ANS fluorescence intensity at 530 nm *vs.* peptide **1** concentrations; (b) kinetic experiment on nucleopeptide **1** aggregation: fluorescence spectra of FITC (121 μM in H_2O; pH = 7.0), 5 (black line), 60 (red) and 120 (grey) min after the addition of 6.5 μM nucleopeptide **1**; inset of Fig. 7b: time dependence of FITC fluorescence intensity after addition of nucleopeptide **1**.

Metal ion binding studies

It is known that nucleobase stacking can occur in the presence of different metal cations such as copper(II) and palladium(II).[15] This interaction is at the basis of supramolecular architectures assembled by the copper(II) binding both in nucleopeptide molecules and in nucleosides or nucleotides.[16–18] Interestingly, copper(II)–peptide complexes can also possess biological activity as reported for the natural tripeptide glycyl-L-histidyl-L-lysine (GHK) which acts as a cell growth factor.[19] In order to investigate the influence of copper(II) ions on the supramolecular networks based on the dithymine tetraserine, we performed UV experiments in which the absorbance of a 10 μM solution of nucleopeptide, in the presence of phosphate buffer at pH 7.5, was recorded after adding increasing amounts of a $CuCl_2$ solution. As can be seen in Fig. 8 the UV absorbance due to the thymine moieties

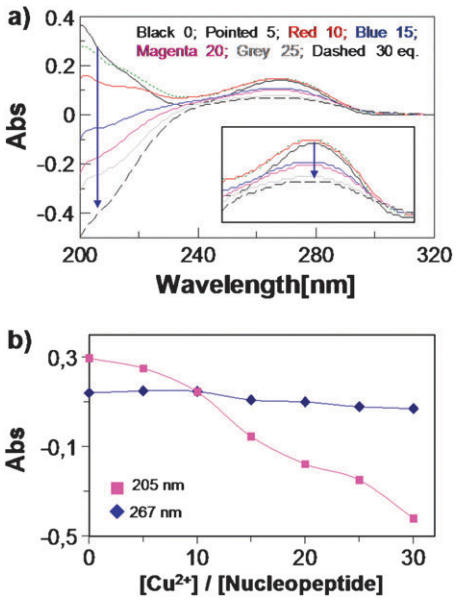

Fig. 8 (a) UV absorbance of a 10 μM solution of nucleopeptide **1**, (10 mM phosphate buffer at pH 7.5, 25 °C) in the presence of 0, 5, 10, 15, 20, 25 and 30 equivalents of Cu(II), (b) plot of UV absorbance at 205 (pink) and 267 (blue) nm *vs.* Cu(II)/nucleopeptide concentration ratio.

decreases in the presence of copper(II) cations, confirming also in this case an interaction of the metal with the nucleobases which causes a clear stacking effect. In other words the L-serine nucleopeptide, which is intrinsically able to form supramolecular networks, shows also a nucleobase stacking effect in the presence of copper(II) which is an interesting feature which may open up new possibilities for the fabrication of nucleopeptide–metal hybrid materials governed by noncovalent nucleopeptide–metal ion interactions.

Biological studies—DNA binding evaluation

DNA binding ability of the L-serine-based nucleopeptide was evaluated by CD spectroscopy. Firstly, the CD profile of the single strand nucleopeptide **1** 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 **1** in the range 190–240 nm at different temperatures (5, 10 and 25 °C) no significant α-helical contribution was detected (data not shown).

Regarding the binding with DNA, CD experiments were performed at 5 °C in a tandem cell recording the "sum" spectrum of the separated components and the "mix" spectrum, recorded after mixing the two ligand solutions, relative to 1 equivalent in nucleobase of dA_{12} with respect to nucleopeptide **1**. The slight difference observed between the "sum" (green line) and "mix" (blue) CD spectra suggested a very weak interaction between the nucleopeptide and DNA (Fig. S5, ESI†). On the other hand, no interaction with RNA (A_{12}, polyA and polyU) was revealed by analogous experiments in the same experimental conditions (data not shown).

Human plasma stability assay

The enzymatic resistance of the novel nucleopeptide was investigated by incubating the oligomer in 40% human plasma at 37 °C and analyzing, by RP-HPLC, samples withdrawn from the reaction mixture at various times (Fig. 9). By analyzing the results of the stability assay, it can be deduced that the nucleopeptide is biodegradable and is more resistant than a natural oligonucleotide, presenting a $t_{1/2}$ of about 3 hours.

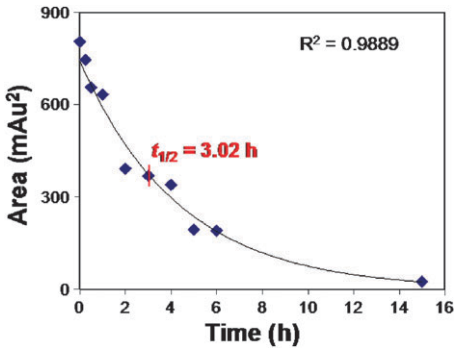

Fig. 9 Area *vs.* time profile relative to the human serum stability assay. In 40% human plasma (T = 37 °C) the dithymine tetrapeptide presents a $t_{1/2}$ = 3.02 h.

Experimental section

Abbreviations

The following abbreviations are used: ANS (8-anilino-1-naphthalenesulfonic acid); DCC (1,3-dicyclohexylcarbodiimide); DCM (dichloromethane); DCU (1,3-dicyclohexylurea); DIPC (*N*,*N*′-diisopropylcarbodiimide); DIPEA (*N*,*N*′-diisopropyl-ethylamine); DMAP (4-dimethylaminopyridine); DMF (*N*,*N*-dimethylformamide); DMSO-d_6 (deuterated dimethyl sulfoxide); FITC (fluorescein isothiocyanate); Fmoc (9-fluorenyl-methoxycarbonyl); MBHA (4-methylbenzhydrylamine); NMP (4-methylpyrrolidone); PyBop (benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate); TCH_2COOH (thymin-1-yl acetic acid); TFA (trifluoroacetic acid); TIS (triisopropyl silane).

Chemicals

Fmoc-L-Ser(tBu)-OH and PyBop were purchased from Novabiochem. Anhydroscan DMF and NMP were from LabScan. Piperidine was from Biosolve. CH_3CN for HPLC chromatography and acetic anhydride were from Reidel-de Haën. TCH_2COOH, TFA, Rink-MBHA-amide resin were from Fluka. DCM and TFA (for HPLC) were from Romil. ANS, deuterated DMSO, DCC, DIPC, DIPEA, DMAP, FITC and TIS were from Sigma-Aldrich. Diethyl ether was from Carlo Erba. dA_{12} was from Biomers.

Apparatus

^{1}H NMR and ^{13}C NMR spectra were recorded at 25 °C on a Varian unity 400 MHz spectrometer. Chemical shifts (δ) are given in parts per million (ppm). Proton chemical shifts were referenced to residual CHD_2SOCD_3 (δ = 2.49, quin) signals. ^{13}C NMR chemical shifts were referenced to the solvent (CD_3SOCD_3: δ = 39.5, sept).

Centrifugations were performed on a Z 200 A Hermle centrifuge (4 min at 4000 rpm).

Products were analysed and characterized by LC-MS 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^{-1}. Semi-preparative purifications were performed on a Hewlett Packard/Agilent 1100 series HPLC, equipped with a diode array detector, by using a Phenomenex Juppiter 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 were lyophilized in a FD4 Freeze Dryer (Heto Lab Equipment) for 16 h.

Circular dichroism (CD) spectra were obtained on a Jasco J-810 spectropolarimeter, while, ultraviolet (UV) spectra were recorded on a UV-Vis Jasco model V-550 spectrophotometer, both equipped with a Peltier *ETC*-505T temperature controller, using an absorption Hellma quartz cell with light paths of 1 cm and 1 mm, and a Hellma-238-QS tandem quartz cell (2 × 0.4375 cm). Quantification of the purified oligomer was performed by UV measurements (T = 80 °C, absorbance value at λ = 260 nm) of **1** dissolved in MilliQ water: the epsilon value used (17.2 mM^{-1}) was calculated using the molar extinction coefficient of the thymine PNA monomer (8.6 mM^{-1}). Fluorescence experiments were performed on a JASCO model FP-750 spectrofluorimeter equipped with a Peltier temperature controller, using a micro fluorescence cell (Hellma, Suprasil quartz, 10 × 2 mm light path, 0.5 ml volume). For light scattering a MiniDAWN Treos spectrometer (Wyatt Instrument Technology Corp.) equipped with a laser operating at 658 nm was used in a batch mode (off-line).

Solid phase synthesis of nucleopeptide 1

The synthesis was carried out in short PP columns (4 ml) equipped with a PTFE filter, a stopcock and a cap on a Rink-amide resin using the peptide Fmoc chemistry. The tetrapeptide H-[Ser(T)-Ser]$_2$-NH_2 (**1**, Fig. 1) was assembled on Rink-amide–NH_2 resin (0.5 mmol g^{-1}, 40 mg, 20 µmol) by alternatively coupling Fmoc-L-Ser-OH (0.52 M in DMF dry, 9 eq., 381 µl) and the commercial Fmoc-L-Ser(tBu)-OH (0.52 M in DMF dry, 9 eq., 381 µl), and using PyBOP (0.52 M in NMP dry, 9 eq., 381 µl)/DIPEA (18 eq., 61 µl) as the activating system (762 µl DMF/NMP, 1 : 1 v/v; for 20 min). Fmoc deprotection of the amino groups with 20% piperidine in DMF for 15 min followed each coupling step, and by the UV Fmoc test (ε_{301} = 7800) it was possible to evaluate the incorporation yields of each serine unit. No capping step was performed to avoid the acetylation of the free serine hydroxyl groups. Before the final Fmoc removal, the free hydroxyl groups were esterified with the nucleobase acetic acid: to a stirred solution of TCH_2COOH (400 µmol, 10 eq. per OH, 74 mg) in NMP (600 µl) at 0 °C, a solution of DCC (440 µmol, 11 eq. per OH, 91 mg)/DMAP (20 µmol, 0.5 eq. per OH) in NMP (700 µl) was added under Ar atmosphere. After 1 h, the white precipitate of DCU was removed by filtration in a dry-box, and the yellow filtrate solution containing the activated nucleobase acetic acid was added to the pre-swelled resin and allowed to react for 3 h. The UV test of the final Fmoc revealed an overall yield for the

tetrapeptide backbone of about 67%. Subsequently, oligomer **1** was detached from the resin by TFA/TIS/H_2O (95 : 2.5 : 2.5) treatment and recovered by precipitation with cold diethyl ether, centrifugation and lyophilization. The crude material was purified by semipreparative HPLC on a C_{18} column using a linear gradient of 5% (for 5 min) to 30% B′ in A′ over 30 min: t_R = 10.6 min; UV quantification of the purified product gave 4.2 μmol of **1** (2.9 mg; 21% yield). *LC-ESIMS characterization* (Fig. S2, ESI†). Method: 5% (5 min) to 40% B in A over 10 min, t_R = 9.32 min; *m*/*z*: 1397.18 (found), 1396.25 (expected for $[2 \times (C_{26}H_{35}N_9O_{14}) + H]^+$); 720.61 (found), 722.59 (expected for $[C_{26}H_{35}N_9O_{14} + Na]^+$); 699.27 (found), 698.63 (expected for $[C_{26}H_{35}N_9O_{14} + H]^+$).

Synthesis of the Fmoc-L-Ser(T)-OH monomer (5)

The nucleobase-containing monomer was synthesized starting from the commercially available Fmoc-L-Ser(tBu)-OH (**3**, Fig. 2). About 100 mg of **3** (0.261 mmol) were treated with TFA/DCM 1 : 1 (3 ml, RT, 2 h) to selectively remove the *tert*-butyl group. After removal of TFA and DCM under nitrogen stream, the residue was resuspended in 6 ml of cold diethyl ether by the aid of sonication, and Fmoc-L-Ser-OH (**4**) was recovered by centrifugation as a white precipitate (84 mg, 0.257 mmol, almost quantitative yield). *Characterization of Fmoc-L-Ser-OH 4*. LC-ESIMS-method: 15% (5 min) to 95% B in A over 15 min, t_R = 12.50 min; *m*/*z*: 983.82 (found), 983.03 (expected for $[3 \times (C_{18}H_{17}NO_5) + H]^+$); 678.29 (found), 677.67 (expected for $[2 \times (C_{18}H_{17}NO_5) + Na]^+$); 656.59 (found), 655.69 (expected for $[2 \times (C_{18}H_{17}NO_5) + H]^+$); 350.83 (found), 350.33 (expected for $[C_{18}H_{17}NO_5 + Na]^+$); 329.55 (found), 328.35 (expected for $[C_{18}H_{17}NO_5 + H]^+$) (Fig. S3, ESI†). NMR-δ_H (400 MHz, $CDCl_3$) 7.72–7.24 (8H, m, aromatic CH Fmoc), 6.25 (1H, d, $^3J_{H,H}$ = 7.2 Hz, CON*H*), 4.51–4.12 (4H, m, FmocCH-CH_2, FmocC*H*-CH_2, CH_{alpha}), 4.10–3.89 (2H, bdd, CH_2CH_{alpha}); δ_C (100 MHz, $CDCl_3$) 173.6, 157.0, 144.1, 141.6, 128.1, 127.5, 125.6, 120.3, 67.6, 63.3, 56.4, 47.4. Subsequently, compound **4** (84 mg, 0.257 mmol) was dissolved in 1 ml DMF and coupled with thymine acetic acid (1.5 eq., 71 mg), which was previously preactivated with DIPC (1.5 eq., 60 μl)/DMAP (cat., *ca.* 1 mg) in DMF (1 ml), at room temperature (Fig. 2). After 21 h, the reaction was quenched by adding water, freezing and lyophilizing the mixture. The crude material was resuspended in 20% aqueous CH_3CN and purified by semipreparative HPLC on a C_{18} column using a linear gradient of 20% (5 min) to 80% B in A over 20 min; t_R = 19.5 min. The desired product **5** was obtained in 30% yield (39 mg, 0.079 mmol). *Characterization of Fmoc-L-Ser(T)-OH 5*. LC-ESIMS-method: 15% (5 min) to 95% B′ in A′ over 15 min; t_R = 12.82 min; *m*/*z*: 987.83 (found), 987.97 (expected for $[2 \times (C_{25}H_{23}NO_8) + H]^+$); 532.46 (found), 532.58 (expected for $[C_{25}H_{23}NO_8 + K]^+$); 517.71 (found), 516.47 (expected for $[C_{25}H_{23}NO_8 + Na]^+$); 494.82 (found), 495.49 (expected for $[C_{25}H_{23}NO_8 + H]^+$) (Fig. S4, ESI†). NMR-δ_H (400 MHz, DMSO-d_6): 11.39 (1H, s, N*H* thymine), 7.90–7.32 (10H, m, aromatic C*H* Fmoc, Fmoc-N*H*, C*H* thymine), 4.49 (2H, d, FmocCH-CH_2), 4.48–4.01 (6H, m, NCH_2CO, FmocC*H*-CH_2, CH_{alpha}, and CH_2CH_{alpha}), 1.73 (3H, s, CH_3 thymine); δ_C (100 MHz, DMSO-d_6): 174.6, 172.12, 168.25, 160.0, 154.9, 147.7, 145.4, 144.7, 131.6, 131.1, 129.3, 124.1, 112.6, 69.9, 68.17, 56.8, 52.1, 15.9.

Solid phase synthesis of nucleopeptide 1 by using monomer 5

The tetrapeptide H-[Ser(T)-Ser]$_2$-NH_2 (**1**, Fig. 3) was assembled on Rink-amide–NH_2 resin (0.5 mmol g^{-1}, 20 mg, 10 μmol) by alternatively coupling Fmoc-L-Ser(T)-OH (0.2 M in NMP, 5 eq., 250 μl) and the commercial Fmoc-L-Ser(tBu)-OH (5 eq., 19 mg), and using PyBOP (5 eq., 26 mg)/DIPEA (10 eq., 17 μl) as the activating system in NMP (about 400 μl for 20 min). Capping was performed with 20% $(Ac)_2O$/5% DIPEA for 10 min, while Fmoc deprotection of the amino groups was obtained with 20% piperidine in DMF for 15 min. The incorporation yields of each Fmoc-L-Ser(T)-OH monomer (**5**) were of about 80%. The overall oligomer yield, calculated on the basis of the UV Fmoc test, was of about 50%. Cleavage from the solid support and purification of oligomer **1** were performed as described before. ESI-MS characterization confirmed the identity of the nucleopeptide. UV quantification of the purified product gave 5 μmol of **1** (40% yield).

CD studies

All the CD spectra were recorded using the following parameters: scanning speed, 50 nm min^{-1}; data pitch, 2 nm; band width, 2 nm; response, 4 s; 7 accumulations.

Nucleic acids binding studies were performed at 5 °C in 10 mM phosphate buffer (pH 7.5), using a tandem cell. CD spectra relative to the self-assembly studies of **1** were performed at 10 °C in 10 mM phosphate buffer (pH 7.5), using a 1 mm quartz cell. For each nucleopeptide concentration, various CD spectra were collected until the system reached equilibrium.

Human plasma stability assay

180 μl of nucleopeptide **1** solution (92 μM) was added to 120 μl of 100% Human Plasma (HP) in a micro-vial, and the mixture was incubated at 37 °C, resulting in a final 55 μM concentration of **1** and 40% HP. Aliquots (30 μl) of the reaction mixture were withdrawn at various times (0, 15, 30 min, and 1, 2, 3, 4, 5, 6, 15 h), added to a solution of 20% CH_3CN in H_2O (0.1% TFA), left 2 min at 85 °C, and analyzed by RP-HPLC using a linear gradient of 5 (3 min) to 21% CH_3CN (0.1%TFA) in H_2O (0.1%TFA) over 9 min (1.0 mL min^{-1}, at 45 °C).

Static light scattering

A stock solution of nucleopeptide was filtered through a 0.02 μm Millex syringe driven filter unit (Millipore, Bedford, MA). After dilution samples containing the nucleopeptide were prepared in 10 mM phosphate buffer, pH 7.5. All measurements were registered in triplicates for 2 min acquisition time. The hydrodynamic radius (Rh) of the scattering molecules was derived, using the ASTRA software, from the diffusion coefficient using the Einstein–Stokes equation.

Fluorescence

Fluorescence aggregation studies with ANS were carried out using the following instrument parameters: scan type, fluorescence emission; excitation wavelength, 380 nm; emission wavelength, 400 nm; excitation and emission slits, 5 nm; recording speed 125 nm min^{-1}. Emission spectra of a 430 μl solution ANS alone (30 μM in 10 mM phosphate buffer, pH = 7.5), and after successive additions (7.8 μl each) of nucleopeptide **1** (550 μM), were collected. The changes in emission were tracked until the system reached equilibrium (about 90 min). Experiments were performed at both 25 and 10 °C giving the same results reported in Fig. 7a. The kinetic experiments on nucleopeptide **1** aggregation were performed using the following instrument parameters: scan type, fluorescence emission; excitation wavelength, 485 nm; emission wavelength, 490 nm; excitation and emission slits, 2.5 nm; recording speed 125 nm min^{-1}. To a 121 μM FITC solution in H_2O (pH = 7.0), 5 μl of **1** (550 μM) were added, obtaining a nucleopeptide concentration of 6.5 μM. Fluorescence spectra were collected at the following times: 0, 5, 20, 60, 90, 120, 180 min. Experiments were performed at both 25 and 10 °C giving the same results reported in Fig. 7b.

Conclusions

In conclusion, we realized by two different synthetic strategies a dithymine tetrapeptide (**1**, Fig. 1), made of both thymine-containing and unfunctionalized L-serine units alternated in the sequence. Our novel analogue showed a good solubility in water, a desirable property for a peptide-like oligonucleotide mimetic. We demonstrated by the above described studies that this novel analogue of TpT DNA dinucleoside monophosphate can form supramolecular architectures, containing a hydrophobic core able to incorporate poorly water-soluble molecules, which are modified by metal ion interaction. Furthermore, the nucleopeptide is biodegradable and is able to interact very weakly with complementary DNA. The preliminary findings of the present work encourage us to further study the alternated L-serine/L-nucleoserine nucleopeptides, obtainable in the solid phase with an appropriate sequence and number of bases, as novel materials that could be beneficial in the biomedical research as drug delivery agents.

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