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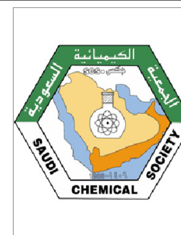

ORIGINAL ARTICLE

Synthesis, self-assembly-behavior and biomolecular recognition properties of thyminyl dipeptides

Giovanni N. Roviello [a,*], Giorgia Oliviero [b], Antonella Di Napoli [a], Nicola Borbone [c], Gennaro Piccialli [c]

[a] *CNR, Istituto di Biostrutture e Bioimmagini – (Via Mezzocannone Site and Headquarters), 80134 Napoli, Italy*
[b] *University of Napoli Federico II, Department of Molecular Medicine and Medical Biotechnology, Via Sergio Pansini, 5, 80131 Napoli, Italy*
[c] *University of Napoli Federico II, Department of Pharmacy, Via Domenico Montesano, 49, 80131 Napoli, Italy*

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Abstract This article describes the synthesis of Thy-(Phe-Phe) and Thy-(Tyr-Tyr), two thymine-bearing dipeptides based on L-phenylalanine and L-tyrosine, the circular dichroism (CD), UV and dynamic light scattering (DLS) characterization of their self-assemblies, and a CD study of their interaction with nucleic acids (using homoadenine DNA and RNA) and serum proteins (utilizing BSA as a model protein). DLS studies, alongside with CD and UV investigations conducted on aqueous solutions of the derivatives under different concentration and temperature conditions, showed the formation of extensive molecular architectures with hydrodynamic mean diameters higher than 300 nm, with Thy-(Tyr-Tyr) forming at pH = 7.5 particularly large and stable networks, involving multiple units, connected by H-bonding, aromatic and hydrophobic interactions. Finally, the findings of our study suggested that Thy-(Phe-Phe) and Thy-(Tyr-Tyr), very stable in human serum, were able to bind BSA protein altering its secondary structure.
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1. Introduction

Peptidyl nucleosides are natural structures composed of nucleobases connected in various ways to amino acid-based moieties (Walsh and Zhang, 2011). Among these compounds, nikkomycin Z is an important antifungal drug, while cystocin is endowed with useful anticancer properties, though many other biomedical applications are known for peptidyl nucleosides (Holden et al., 2014; Lee et al., 2003). Structurally, their nature is highly variegated, with many of them sharing the common nucleoside-peptide nature. Nonetheless, even nucleobase-amino acid-containing structures deprived of the sugar core are equally classified as peptidyl nucleosides, like in the case of the antibiotic blasticidin S (Kitamura et al., 2017). Additionally, structures that bear aromatic rings or nucleobases directly connected to amino acid-containing moieties,

* Corresponding author at: Istituto di Biostrutture e Bioimmagini-CNR, Via Mezzocannone 16, 80134 Napoli, Italy.
E-mail address: giroviel@unina.it (G.N. Roviello).
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are present in a variety of natural sources, or have been subject of synthetic efforts (Dolman et al., 2006; Esposito et al., 2015; Mizutani et al., 1996; Roviello et al., 2011a,b; Teta et al., 2013; Weckenmann and Nachtsheim, 2015).

Examples of these compounds, known as nucleo-amino acids or nucleobase amino acids (NBAs), include (S)-willardiine and its analogs (Dolman et al., 2006), which act as potent agonists of AMPA or kainate receptors, as well as the monomers of synthetic nucleobase-containing peptides (Roviello et al., 2007, 2010c, 2011c, 2012; Roviello and Musumeci, 2016a; Roviello et al., 2016b).

Due to their nucleobase-decorated peptide-like nature, but also for being employed in complementary DNA and RNA targeting, these latter largely recall the PNAs realized by Nielsen in 1991, and subsequently involved in numerous applications (Amato et al., 2014a,b; Nielsen et al., 1991; Pinto et al., 2016; Roviello et al., 2009b).

In the context of the new drug discovery, the insertion of NBAs in short peptides, either biologically active or conferring peculiar structural features, could lead up to several therapeutically relevant consequences, including the possibility to bind nucleic acids, interact with proteins, and inhibit the activity of HIV or other viral reverse transcriptases (Roviello et al., 2008, 2009a, 2010a,b, 2016c).

Not less important, NBA insertion into protein structures can improve their biomolecular recognition properties, favoring, e.g., protein–protein, and RNA–protein recognition (Watanabe et al., 2007).

Moreover, nucleobase-decorated dipeptides can be beneficial in building up supramolecular networks, based on the peptidyl nucleoside self-assembly, for drug delivery applications. In fact, literature examples have clearly demonstrated this possibility for self-assembling dipeptides and nucleobase-based materials (Garcia et al., 2015; Naskar et al., 2011; Thota et al., 2016).

Overall, the important role that peptidyl nucleosides have in the field of antimicrobial and anticancer therapy, as well as the possibility to build up new drug delivery systems, exploiting supramolecular networks based on dipeptides and nucleobases, prompted us to investigate the new peptidyl nucleosides depicted in Fig. 1.

These comprise a dipeptide moiety (based on the aromatic amino acids L-phenylalanine and L-tyrosine) connected *via* a short linker to the DNA base (thymine). Interestingly, both amino acids can originate dipeptides whose self-assembly in turn leads to well-characterized three-dimensional architectures (Koley et al., 2015) and, thus, here the corresponding N-thyminyl derivatives were synthesized for the first time in their amide forms and studied as novel self-assembling materials.

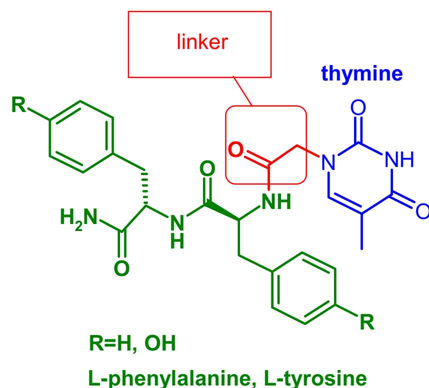

Fig. 1 Schematic representation of the molecular structure of the peptidyl nucleoside analogs realized in the present work and of their natural precursors.

In fact, we aimed to ascertain if the nucleobase-dipeptide conjugated nature of the above-mentioned constructs still caused self-assembly, as well as to evaluate their biomolecular recognition properties. Herein, we investigate both these biologically relevant aspects using UV and CD spectroscopies, DLS and other methodologies, as we describe below in this manuscript.

2. Experimental

2.1. Materials

Fmoc-L-Phe-OH and Fmoc-L-Tyr(tBu)-OH were Inbios, while HATU was Novabiochem. Ethanol was purchased from Fluka; BSA, human serum, piperidine, TCH_2COOH and diethyl ether were Sigma Aldrich. DMF, DIEA, TFA and HPLC solvents were Romil. Poly rA RNA was Fluka and dA_{12} DNA was purchased from Biomers. ^1H NMR and ^{13}C NMR spectra were recorded at 25 °C on Varian 400 MHz or 600 MHz spectrometers. Chemical shifts (δ) are given in parts per million (ppm) and all coupling constants (J) are given in Hz. ^1H NMR chemical shifts are referenced to residual CHD_2OD (δ = 3.30, quin) or CHD_2SOCD_3 (δ = 2.49, quin). ^{13}C NMR chemical shifts are referenced to the solvent (CD_3OD: δ = 49.3, or CD_3SOCD_3: δ = 39.5 ppm). Samples of peptidyl nucleoside analogs underwent LC-MS analysis, on a 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 a MS pump, an autosampler, and a PDA detector, by using a Phenomenex Jupiter 4 μm Proteo 90 Å column (4 μm, 4.6 × 150 mm). We performed the gradient elution at 25 °C (monitoring at λ = 260 nm) 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}. We obtained the analytical chromatograms on a Hewlett Packard/Agilent 1200 series HPLC system, equipped with a diode array detector, and used a Phenomenex Jupiter C18 300 Å (5 μm, 4.6 × 250 mm) column. Also in this case, gradient elution was performed at 25 °C (monitoring at λ = 260 nm), by using a gradient that started with buffer A′ (0.1% TFA in water) and applying buffer B′ (0.1% TFA in acetonitrile) with a flow rate of 1 mL min^{-1}. Peptidyl nucleoside samples were lyophilized in a FD4 Freeze Dryer (Heto Lab Equipment) for 16 h. We performed Dynamic Laser Light Scattering (DLS) studies on a Zetasizer Nano ZS instrument (Malvern Instruments ltd).

2.2. Synthetic procedures

The two analogs based on L-phenylalanine **[Thy-(Phe-Phe)]** and L-tyrosine **[Thy-(Tyr-Tyr)]** were assembled on a Rink Amide MBHA resin (0.65 mmol/g, 77 mg, 50 μmol), that was previously treated with 40% piperidine in DMF (30 min)

for initial Fmoc-removal. More in detail, a solution of Fmoc-L-Phe-OH or Fmoc-L-Tyr(Boc)-OH (250 µmol, 5 equiv.), HATU (250 µmol, 5 equiv.) and DIEA (500 µmol, 10 equiv.) in 0.8 mL of anhydrous DMF was added to the NH_2-resin and the mixture was allowed at room temperature under stirring over 30 min. The same procedure was repeated once again. Afterwards, we removed Fmoc group by treatment with 40% piperidine in DMF over 20 min. Following the same procedure, we introduced the second amino acid to obtain the dipeptide backbones. Thus, the dipeptide-bearing resin was reacted with TCH_2COOH (250 µmol, 5 equiv.), HATU (250 µmol, 5 equiv.) and DIEA (500 µmol, 10 equiv.) in 0.7 mL of DMF. After 30 min, the same procedure was repeated once again. Finally, the thyminyl dipeptides were detached from the solid support by treatment with TFA/H_2O (95/5, v/v) over 2 h and, once the liquid phase was removed under nitrogen flux, the compounds were precipitated by cold diethyl ether treatment, centrifugation and lyophilization. Interestingly, both peptidyl nucleoside analogs were obtained with their C-terminal carboxyl groups in amide form (–$CONH_2$). Samples were analyzed by HPLC, NMR and ESI-MS that all confirmed the identity of the desired samples, both obtained in quantitative yield. **Thy-(Phe-Phe)**: LC-ESIMS *m/z* 478.02 (found), 478.53 (expected for $[C_{25}H_{27}N_5O_5 + H]^+$), 500.11 (found), 500.51 (expected for $[C_{25}H_{27}N_5O_5 + Na]^+$), 955.08 (found), 956.05 (expected for $[2 \times C_{25}H_{27}N_5O_5 + H]^+$); 993.36 (found), 994.15 (expected for $[2 \times C_{25}H_{27}N_5O_5 + K]^+$); NMR δ_H (400 MHz, d_6 DMSO) 11.21 (1H, s, NH thymine), 8.32 (1H, d, J = 6.0, NH amide), 8.05 (1H, d, J = 7.6, NH amide), 7.20–7.01 (11H, m, Ar-Phe, C_6-H), 4.45–4.30 (2H, m, $CH\alpha$), 4.22 (1H, d, J = 16.4, $NCH\alpha H$), 4.15 (1H, d, J = 16.8, $NCH_\beta H$), 2.99–2.89 (2H, m, $CH_\beta' H$), 2.78 (1H, bdd, $CH_\beta' H$), 2.72 (1H, bdd, $CH_\beta' H$), 1.67 (3H, s, CH_3); δ_C (100 MHz, d_6 DMSO) 172.6, 170.4, 166.8, 164.4, 151.0, 142.1, 137.8, 137.5, 129.2, 129.1, 128.1, 128.0, 126.2 (2 carbons), 108.0, 54.1, 53.9, 49.1, 37.5, 34.3, 11.9. **Thy-(Tyr-Tyr)**: LC-ESIMS *m/z* 509.93 (found), 510.53 (expected for $[C_{25}H_{27}N_5O_7 + H]^+$); NMR δ_H (600 MHz, CD_3OD) 7.97–6.66 (10H, m, Ar-Tyr, C_6-H, NH thymine), 4.48–4.43 (2H, m, CH_α), 4.37 (1H, d, J = 16.2, $NCH_\alpha H$), 4.29 (1H, d, J = 16.2, $NCH_\beta H$), 3.09 (1H, dd, J = 5.4, 14.4, $CH_\beta H$), 2.95 (1H, dd, J = 6.0, 14.4, $CH_\beta' H$), 2.84 (1H, dd, J = 9.6, 14.4, $CH_\beta' H$), 2.68 (1H, dd, J = 9.0, 14.4, $CH_\beta' H$), 1.86 (3H, s, CH_3); δ_C (150 MHz, CD_3OD) 176.3, 173.4, 170.2, 167.1, 157.6, 143.7, 131.6, 131.5, 129.6, 129.1, 126.7, 126.2, 116.6, 116.5, 111.5, 57.3, 56.5, 51.6, 38.4, 31.9, 12.5.

2.3. CD studies

Circular dichroism (CD) studies were conducted in analogy to other literature examples (Moccia et al., 2010). In particular, we obtained the CD spectra at 10 °C on a Jasco J-715 spectropolarimeter equipped with a Peltier PTC-423S/15 with a Tandem Hellma (2 × 0.4375 cm) quartz cell or a quartz cell with a light path of 1 cm; ultraviolet (UV) spectra were recorded on a UV–Vis Jasco model V-550 spectrophotometer equipped with a Peltier ETC-505T temperature controller. CD spectra were recorded in the 200–275 (BSA interaction study), 207–320 or 198–320 nm wavelength range. CD spectra for BSA and peptidyl nucleoside/BSA complex were obtained in a Tandem cell by using a 13 mg/L concentration of protein.

3. Results and discussion

3.1. Solid phase synthesis of the peptidyl nucleoside analogs

The artificial peptidyl nucleoside analogs were obtained by solid phase synthesis following the synthetic procedure described in Fig. 2.

The route comprises two main steps: first, we prepared the dipeptide backbones (Phe-Phe, Tyr-Tyr), that finally we functionalized *via* amidation with a thymine-bearing acetic acid. After detachment from the resin under acidic conditions, the two constructs were analyzed and characterized by NMR and ESIMS. In the Supporting Information of this manuscript, we report the LC-ESIMS analysis of both thyminyl dipeptides (Figs. S1 and S2) that confirmed the identity of both compounds. The resulting thyminyl dipeptides [Thy-(Phe-Phe) and Thy-(Tyr-Tyr)] are simplified peptidyl nucleosides in which the sugar moiety is replaced by a peptide segment.

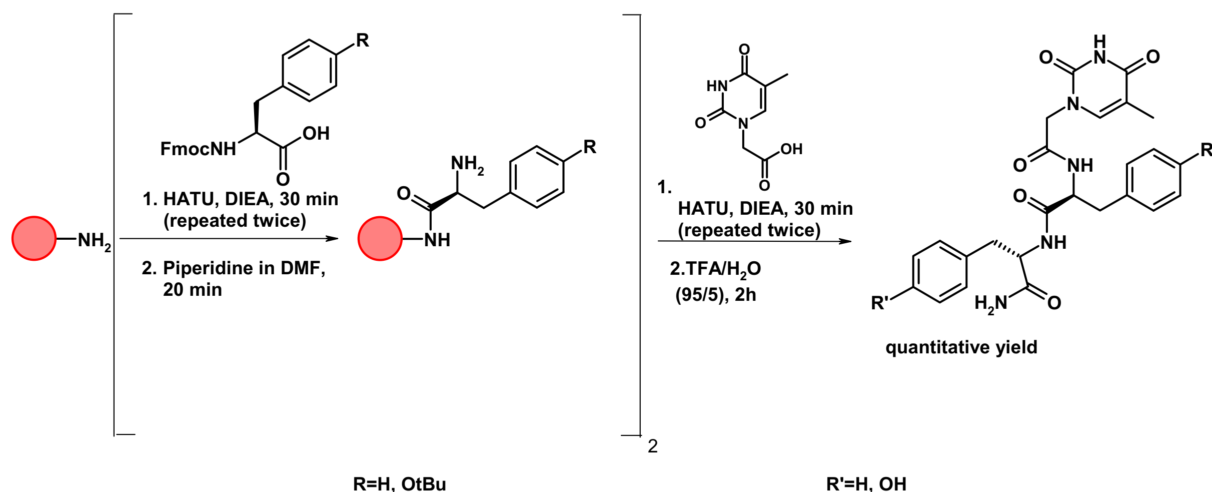

Fig. 2 Schematic representation of the synthesis of the peptidyl nucleoside analogs based on L-phenylalanine and L-tyrosine.

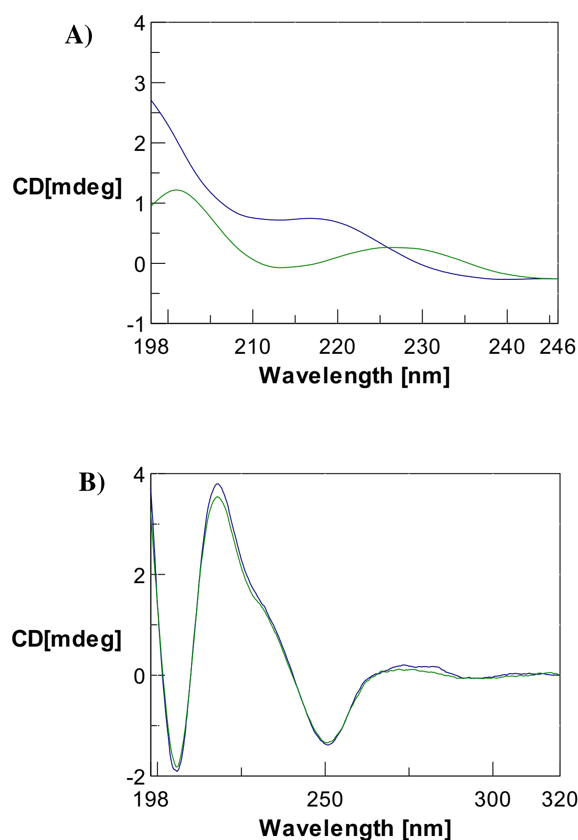

Fig. 3 (A) CD spectra recorded at 10 °C for solutions of the derivatives based on L-phenylalanine (▬) and L-tyrosine (▬) (C = 10 μM) in 10 mM phosphate buffer (pH = 7.5). (B) Sum (▬) and complex (▬) CD spectra of L-Phe-based derivative (8 nmol of T base) + dA_{12} DNA (8 nmol of A base) in 10 mM phosphate buffer (pH = 7.5, 10 °C).

3.2. Nucleic acid interaction studies

Afterwards, we made use of CD spectroscopy in order to study the structural properties of thyminyl dipeptides, and investigate their interaction with nucleic acids. First, we examined the CD spectra of both uncomplexed nucleopeptides and found that while no band was present in the near-UV spectral region, Thy-(Phe-Phe) and Thy-(Tyr-Tyr) presented positive bands centered approximately at 220 and 230 nm, respectively (Fig. 3A). Moreover, by using a two-chamber quartz cuvette, we compared the CD spectra relative to the sum of the separated solutions of thyminyl dipeptides ($C_{Thymine}$ = 5 μM) and nucleic acids (dA_{12} DNA and poly rA RNA; $C_{Adenine}$ = 5 μM; 10 mM phosphate buffer, pH 7.5; T = 10 °C) with those obtained after the above-mentioned solutions were mixed. No significant change in CD profiles was detected in these experiments, but the case of Thy-(Phe-Phe)/DNA. In this case, changes in the positive bands of DNA CD spectrum upon addition of peptidyl nucleoside testify a certain Thy-(Phe-Phe)/DNA binding (Fig. 3B). In other terms, different substitutions (e.g. R = OH versus H) on the phenyl rings of the peptidyl nucleosides cause different effects on the overall DNA-binding ability of the derivatives, with the more hydrophobic compound being more prone to such biomolecular recognition.

Overall, since only minor CD changes were observed in our binding assays, we can conclude that the peptidyl nucleoside analogs are not able to provoke significant structural variations in single-stranded DNA and RNA. This can be associated to the self-assembly behavior of both thyminyl-dipeptides in aqueous solution, as demonstrated and described by us in the following sections, that prevents them from freely binding both nucleic targets.

Thus, it was not by chance that Thy-(Tyr-Tyr), endowed with the greater tendency to form supramolecular networks (reinforced by additional H-bonding due to the hydroxyl moieties of L-tyrosine residues), was practically ineffective in binding nucleic acids as seen in the case of' dA_{12} DNA and poly rA RNA.

3.3. The peptidyl nucleoside analogs form supramolecular networks

The main goal of this work was to realize molecular networks, formed by non-covalent polymerization of the two already described compounds, i.e. self-assemblies that could be interesting tools for drug delivery applications. In fact, we were driven in this investigation by the evidence that other short peptide structures similar to ours were effective in forming supramolecular networks (Koley et al., 2015; Naskar et al., 2011; Thota et al., 2016). Not of less importance, the presence of a DNA nucleobase, element involved in other successful supramolecular networking systems (Garcia et al., 2015), was another encouraging feature that reinforced our hypothesis. Therefore, we have investigated the formation of supramolecular networks also in the case of our nucleobase-dipeptide conjugates, using to this scope UV and CD spectroscopies and DLS, as described below. First, we recorded a CD spectrum of the solutions of both derivatives at a 48 μM concentration under controlled pH and temperature conditions (pH 7.5 and 10 °C). In CD of Thy-(Phe-Phe) we noticed no CD signal around 260 nm, but only a positive band at about 220 nm (fx1; Fig. 4A). in the case of Thy-(Tyr-Tyr) we could observe a positive band centered at about 230 nm (fx1; Fig. 4B), but still no CD signals were detected at 260 nm.

Thus, we examined the CD spectral changes upon variation of temperature heating the same solutions of two peptidyl nucleoside analogs. More in detail, we recorded for both derivatives further CD spectra at the following temperatures: 30, 50 and 70 °C. In both cases, we observed a decrease of spectral intensity as a consequence of the heating of the solutions, as shown in Fig. 4 (A and B).

This can be better evidenced by plotting CD values recorded at 220, 236 and 270 nm (Fig. 5a–c), for Thy-(Phe-Phe), and at 228 and 214 nm, for Thy-(Tyr-Tyr) (Fig. 6a and b). The CD variation and, more in particular, the decrease of CD band intensities caused by temperature increase, sustained our hypothesis of a supramolecular networking involving multiple peptidyl nucleoside units held together by H-bonding, aromatic interactions and other weak interaction forces. In fact, the decrease of CD intensity can be interpreted in terms of a rearrangement of these networks due to the heating of the solutions.

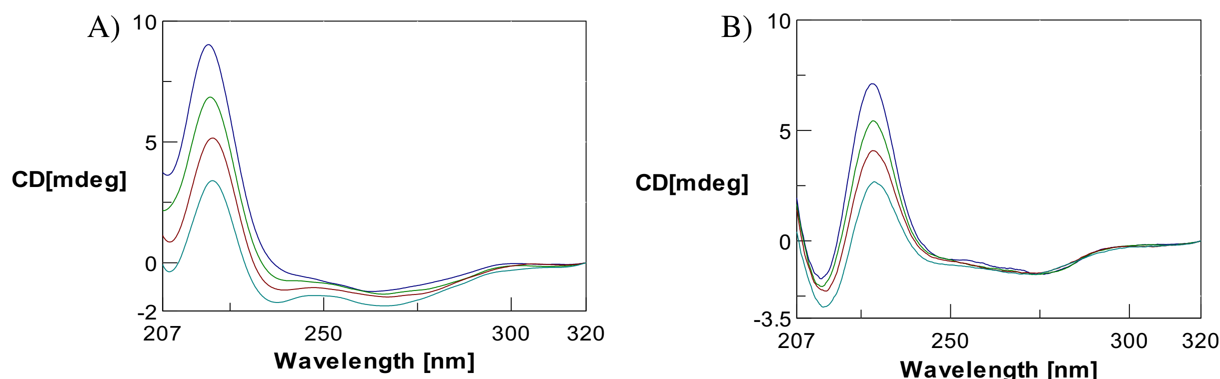

Fig. 4 CD spectra recorded at 10 (blue), 30 (green), 50 (red) and 70 (light blue) °C for solutions of the derivatives based on L-phenylalanine (A) and L-tyrosine (B) (C = 48 μM, V = 3 mL) in 10 mM phosphate buffer (pH = 7.5).

A further confirmation of the above hypothesis came from a second CD experiment performed at a fixed temperature (10 °C) on solutions of the two constructs in which their concentrations were varied in the 6.4–32.0 μM range (Fig. 7). Interestingly, a negative induced CD band at about 250 nm and a positive one at about 300 nm emerged in the case of Thy-(Phe-Phe) upon increasing the concentration of the compound from 6.4 to 32.0 μM (Fig. 7A). On the other hand, Thy-(Tyr-Tyr) furnished a negative induced CD band at about 275 nm under the same experimental conditions as a consequence of the same concentration increase (Fig. 7B).

Importantly, these concentration-dependent CD changes, especially evident at 250 and 295 nm in the case of Thy-(Phe-Phe) (Fig. 8A), and at 275 nm in the case of Thy-(Tyr-Tyr) (Fig. 8B), are in favor of the already-cited hypothesis of non-covalent polymeric systems formed under our experimental conditions.

After all, the formation of supramolecular architectures in our two systems was expected by us, as other nucleobase and dipeptide-based supramolecular systems were described in the literature (Garcia et al., 2015; Naskar et al., 2011; Thota et al., 2016), though this property had never been explored before in the case of nucleobase-containing dipeptides.

Furthermore, we analyzed by Dynamic Laser Scattering (DLS) the solutions of these peptidyl nucleosides, to obtain a definitive confirmation of the self-assembly, as well as a further information on the networks revealed by our CD study. Thus, we performed our DLS experiments in triplicate on 300 μM solutions of our peptidyl nucleoside analogs in 9 mM phosphate buffer (pH 7.5, T = 30 °C).

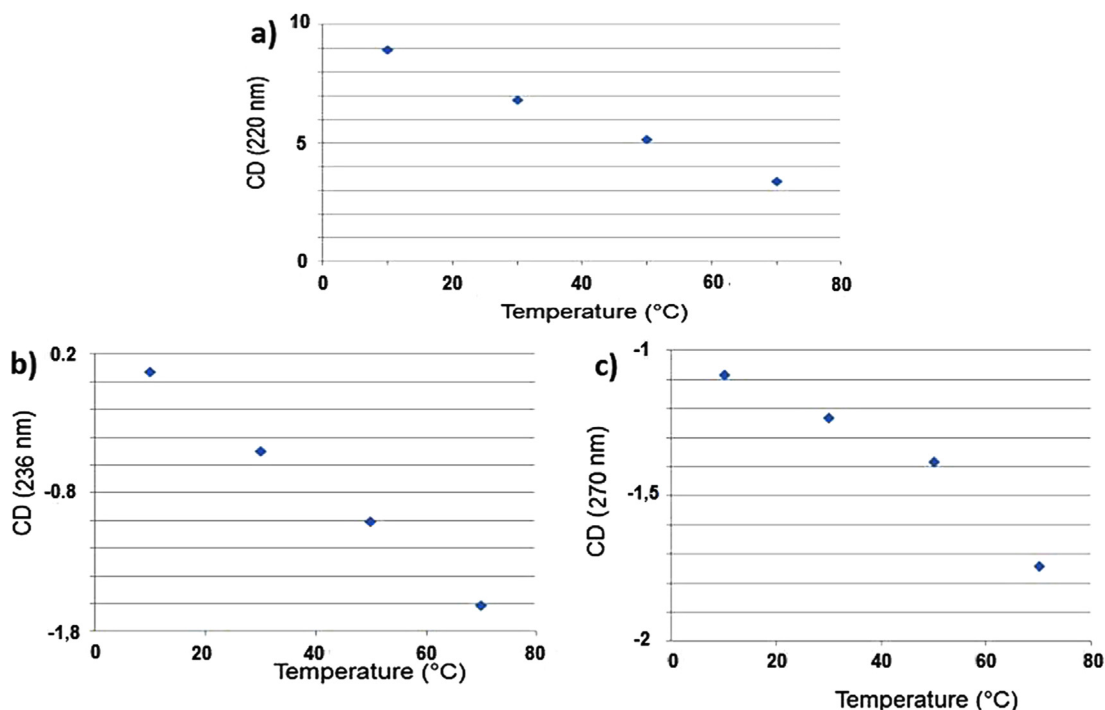

Fig. 5 Variation of the CD values recorded at 220 (a), 236 (b) and 270 (c) nm for a solution of L-phenylalanine-based compound in 10 mM buffer (pH 7.5) as function of temperature (10, 30, 50 and 70 °C).

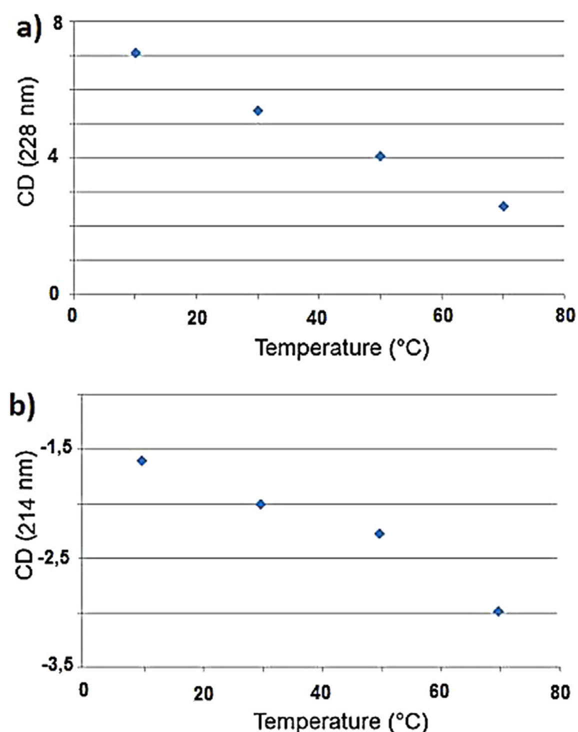

Fig. 6 Variation of the CD values recorded at 228 (a) and 214 (b) nm for a solution of L-tyrosine-based compound in 10 mM buffer (pH 7.5) as function of temperature (10, 30, 50 and 70 °C).

Both thyminyl dipeptides were prone to self-assemble at 30 °C with the formation of aggregates that corresponded to aggregates with hydrodynamic radii (R_H) of 164 ± 10 nm [Thy-(Phe-Phe)], or higher than 200 nm [Thy-(Tyr-Tyr)]. With regards to Thy-(Tyr-Tyr), the hydroxyl moieties probably account for the larger size of the supramolecular networks found by us, with respect to those formed by the phenylalanine-containing molecules. Far-UV CD spectra of our compounds and hydrodynamic radii are similar to those previously reported for aromatic residue containing aggregating to nanovesicular systems (Mishra et al. 2008). Further details on the supramolecular networking came from UV spectroscopy. In fact, as it can be seen in Fig. 9, UV absorption of the two compounds at about 270 nm in phosphate buffer solution changed passing from 30 to 70 °C. In particular, we observed a significant increase of absorbance in the case of Thy-(Phe-Phe) solution and a hypochromic effect in the case of Thy-(Tyr-Tyr).

Similarly, also CD studies (Fig. 4) had revealed a significant variation of the signal at 270 nm in the case of Thy-(Phe-Phe) with a $\Delta CD_{(270\ nm)}$ = 0.52 mdeg upon heating the solution from 30 to 70 °C, whereas in the case of Thy-(Tyr-Tyr) the variation was much more modest, with a $\Delta CD_{(270\ nm)}$ = 0.02 mdeg.

These UV and CD evidences suggest a very thermally stable self-assembly in the case of Thy-(Tyr-Tyr), due to additional H-bonding possibilities given by hydroxyl moieties, with respect to more flexible networks based on OH-lacking Thy-(Phe-Phe), whose self-assembling behavior at 30 °C had, nevertheless, been demonstrated by our DLS study, revealing a hydrodynamic diameter greater than 300 nm. Heating the solutions in the above-reported temperature range, Thy-(Phe-Phe) networks disaggregated at 70 °C, while Thy-(Tyr-Tyr) self-assemblies were practically untouched or even reinforced after the same thermal treatment.

3.4. Serum protein binding and stability in human serum

Protein-protein interactions or, more in general, protein-associated processes are subjects of several studies aimed at modulating these biomolecular events by using organic molecules or peptides (Russo et al., 2016; Vicidomini et al., 2015).

In this respect, we were interested in studying the interaction of our compounds with proteins in view of their possible employment as protein-targeting drugs, as well as to evaluate their potential to form complexes suitable for drug delivery applications.

To this purpose, we made use of BSA (bovine serum albumin) as a model protein, and performed CD binding experiments in a two-chamber quartz cuvette, placing BSA (0.19 μM, 10 mM phosphate buffer, pH 7.5) in one cell chamber, and Thy-(Phe-Phe) or Thy-(Tyr-Tyr) (8 μM, 10 mM

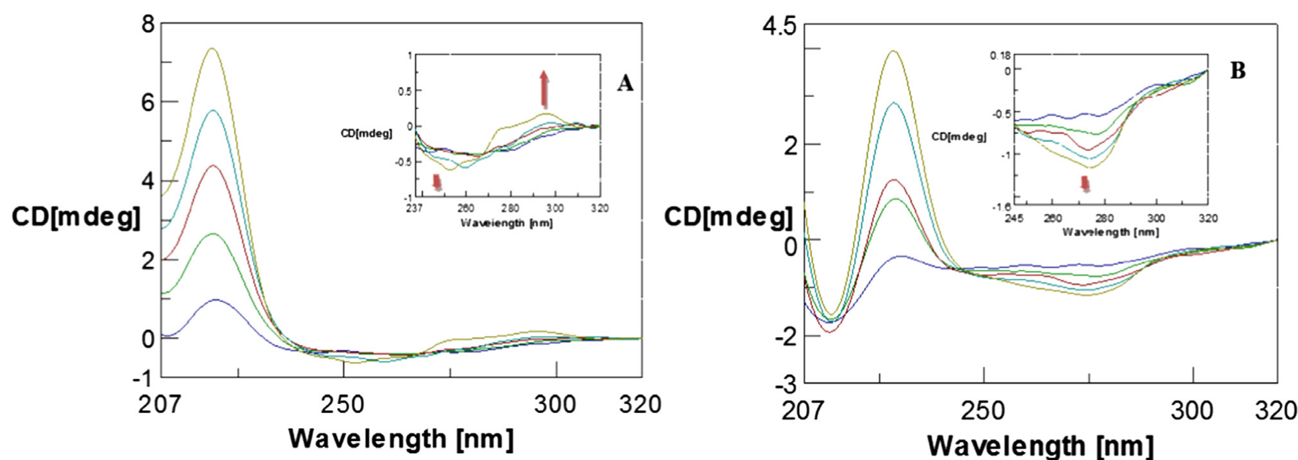

Fig. 7 CD spectra (T = 10 °C) for solutions of Thy-(Phe-Phe) (A) and Thy-(Tyr-Tyr) (B) at variable concentrations (ranging from 6.4 to 32.0 μM) in 10 mM phosphate buffer (pH 7.5).

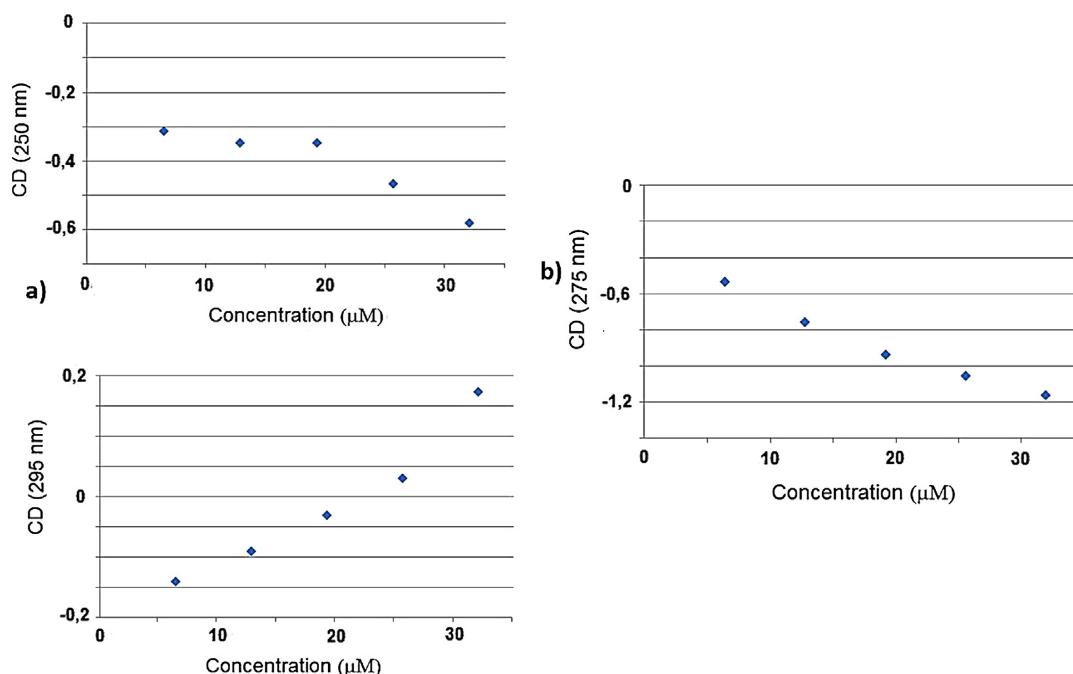

Fig. 8 Variation of the CD values recorded at 250, 295 and 275 nm for solutions of derivatives containing L-phenylalanine (a) and L-tyrosine (b) in 10 mM buffer (pH 7.5) as function of concentration (6.4–32.0 μM).

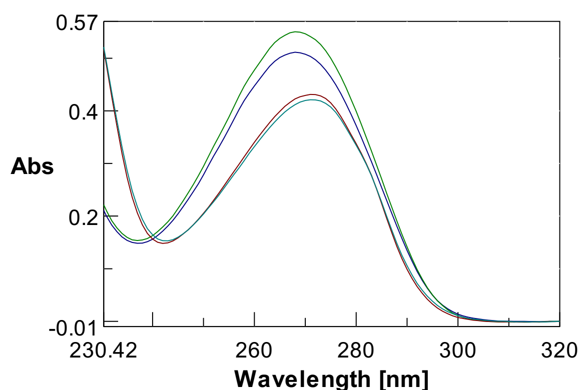

Fig. 9 UV spectra recorded at 30 (▬, ▬) and 70 (▬, ▬) °C for solutions of the derivatives based on L-phenylalanine (upper lines) and L-tyrosine (bottom) (C = 48 μM, V = 3 mL) in 10 mM phosphate buffer (pH = 7.5).

phosphate buffer, pH 7.5) in the other. Subsequently, we recorded the CD spectra before (green) and after (blue lines, Fig. 10) the two separate ligand solutions were mixed.

With regards to Thy-(Tyr-Tyr)/BSA, only weak changes in CD were detected (Fig. 10B), while the variations were significantly more evident in the case of Thy-(Phe-Phe)/BSA (Fig. 10A). In other words, the phenylalanine-based compound was able to bind the protein modifying its secondary structure more effectively than the tyrosine-containing derivative. The Thy-(Phe-Phe)/BSA binding leads to a clear variation of the CD profile in the spectral regions at ~210 nm and 222 nm. This binding provokes, thus, a significant variation of the secondary structure elements of the serum protein, probably due to hydrophobic interactions involving Thy-(Phe-Phe) and apolar regions of BSA. This seems to indicate that the additional H-bonding possibility of Thy-(Tyr-Tyr) is not associated to a higher hybridization propensity of this peptidyl nucleoside with BSA protein, but to the formation of self-assemblies that, as above-reported, are larger and more stable than those formed by Thy-(Phe-Phe).

Furthermore, we evaluated the stability of both peptidyl nucleoside analogs in human serum. We incubated two solutions of Thy-(Phe-Phe) and Thy-(Tyr-Tyr) (C = 0.1 mM) in 90% fresh human serum at 37 °C and analyzed by RP HPLC samples withdrawn from the incubation mixture at several time points (ranging from 0 to 24 h) after their quenching by 7 M urea at 95 °C over 2 min.

The findings of this stability assay (see Fig. S3, Supporting Information) indicate that both thyminyl dipeptides are stable *in sero* under our experimental conditions, as their HPLC peaks (at 17.5 and 14.0 min in case of Thy-(Phe-Phe) and Thy-(Tyr-Tyr), respectively) were found unchanged after 24 h.

3.5. Conclusion

Here we reported on two peptidyl nucleoside analogs [Thy-(Phe-Phe) and Thy-(Tyr-Tyr)] that are based on simple dipeptide structures conjugated to thymine nucleobase through an amide linker. Both compounds were obtained by solid phase synthesis, purified and fully characterized. Extensive CD, UV and DLS studies, described in the present work, demonstrated for both analogs the formation of supramolecular networks, endowed with a larger hydrodynamic diameter in the case of Thy-(Tyr-Tyr), formed by multiple units of thyminyl dipeptides in aqueous solutions, whose structure evolved upon variation of both concentration and temperature, especially in case of Thy-(Phe-Phe).

The additional H-bonding possibilities, presented by the tyrosine-based construct with respect to the phenylalanine

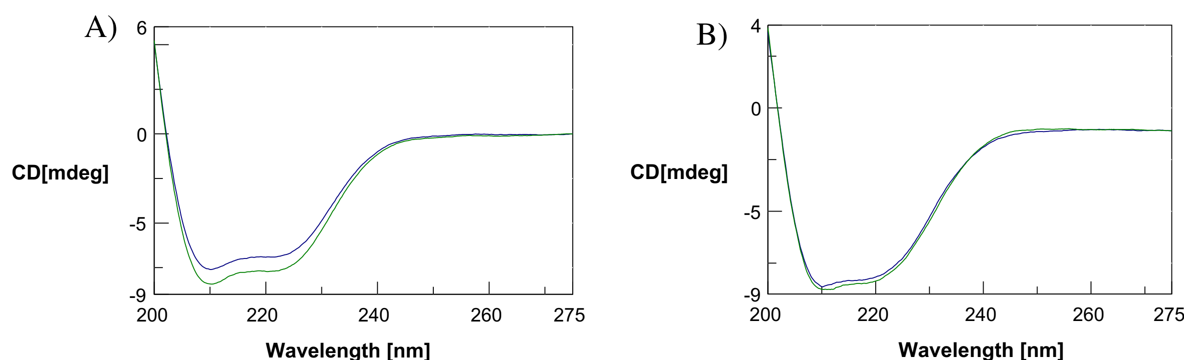

Fig. 10 Sum (——) and complex (——) CD spectra for the systems composed of the peptidyl nucleoside analogs [Thy-(Phe-Phe), A and Thy-(Tyr-Tyr), B] and BSA (0.19 μM) in 10 mM phosphate buffer (pH = 7.5) at 10 °C.

counterpart, seem to favor a more extensive and stable non-covalent polymerization of Thy-(Tyr-Tyr) which is, consequently, less prone to interact with biomolecules. In fact, only Thy-(Phe-Phe) binds dA_{12} DNA, even if at a low extent, and shows a clear BSA-binding as our CD studies have showed, whereas the self-assembly behavior prevents Thy-(Tyr-Tyr) to interact with the biological targets explored by us.

Future efforts will be devoted also to modify the above-presented structures with π-conjugated systems, as promising materials for nonlinear optical applications (Bures et al., 2012). Not secondarily, our thyminyl dipeptides are able to bind proteins and, thus, can be useful in modulating protein-associated processes of therapeutic importance and, finally, due to their structural analogy with the natural peptidyl nucleosides, deserve future scientific efforts in order to evaluate their potential as antimicrobial and anticancer agents for new applications in biomedicine. All these features complete the picture of nucleobase dipeptides as versatile compounds utilizable not only as drugs themselves, in protein-targeting therapeutic approaches, but also to build up serum-stable delivery agents of, e.g., nucleic acid and protein-based drugs.

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Disclosure

The authors report no conflicts of interest in this work.

Appendix A. Supplementary material

This section provides for both peptidyl nucleosides useful data on: (i) LC-ESIMS (positive mode) characterisation; (ii) human serum stability assay (expanded regions of HPLC chromatograms). Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.arabjc.2018.02.014.

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