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ORIGINAL ARTICLE

Thiophenyl-substituted triazolyl-thione L-alanine: asymmetric synthesis, aggregation and biological properties

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Abstract In this work, we report the asymmetric synthesis and characterization of an artificial amino acid based on triazolyl-thione L-alanine, which was modified with a thiophenyl-substituted moiety, as well as in vitro studies of its nucleic acid-binding ability. We found, by dynamic light scattering studies, that the synthetic amino acid was able to form supramolecular aggregates having a hydrodynamic diameter higher than 200 nm. Furthermore, we demonstrated, by UV and CD experiments, that the heteroaromatic amino acid, whose enzymatic stability was demonstrated by HPLC analysis also after 24 h of incubation in human serum, was able to bind a RNA complex, which is a feature of biomedical interest in view of innovative antiviral strategies based on modulation of RNA–RNA molecular recognition.

Keywords Artificial amino acid · RNA · CD · Light scattering

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Introduction

Non-proteinogenic α-amino acids are constituents of many physiologically active peptides, antibiotics and other pharmaceutical preparations (Cativiela et al. 1997). In particular, a great attention was given to heterocyclic analogues of non-proteinogenic α-amino acids which are alien to the human organism due to both their structure and the nature of heteroatoms contained (Katritzky et al. 1984; Harris and Huppatz 1977). Among the possible heterocyclic derivatizations, it is worth to mention the introduction of thiophenyl and triazole moieties, which are important constituents of many biologically and pharmacologically active compounds, such as anti-inflammatory, antibacterial, anticancer and antifungal drugs (Kumar et al. 2004; Queiroz et al. 2006; Thomson et al. 2006; Desai et al. 2008; Shashikant et al. 2009). Undoubtedly, the introduction of such heterocyclic moieties into the side-chain of optically active amino acids and peptides can result in novel biologically and pharmacologically-active compounds of clear biomedical importance (Simeone et al. 2011, 2012).

In the last decades, a crucial importance has been attributed to the realization of hydrogels, as well as water-soluble macromolecular networks, suitable for the incorporation and the release of genes and drugs (Shuai et al. 2005). These systems are non-covalent polymers which are based on one or more types of monomeric units, governed by weak interactions occurring between the subunits. Among the many classes of molecules used to form supramolecular architectures, also peptides and single amino acids with artificial heteroaromatic side chains were previously described (Roviello et al. 2009, 2010a, b, 2011a, b, 2012, 2014).

In the present work, we realized an artificial amino acid (Fig. 1) which bears as a substituent a heteroaromatic

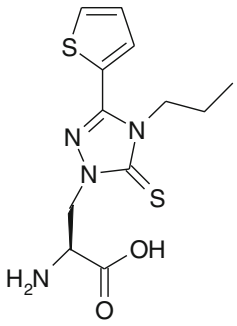


Fig. 1 Molecular representation of the artificial amino acid studied in the present work

moiety, consisting of a triazolyl-thione residue modified with both a thiophenyl and a *n*-propyl groups, elements conferring an apolar nature to the derivative which can be classified, thus, as a non-proteogenic aromatic amino acid.

The introduction of such heteroaromatic moiety into the amino acid is of crucial importance for the possibility to improve the molecular recognition towards targets of biomedical significance, such as nucleic acids, through the interaction with hydrophobic and aromatic moieties present in their structure. Furthermore, the heteroaromatic amino acid shares similarity with nucleobase-modified amino acids, which were previously found able to form supramolecular networks. The present work gives, thus, a description of the asymmetric synthesis, purification and characterization of the above-mentioned amino acid, as well as a study of some biological properties, such as the *in sero* stability and the ability of the artificial molecule to bind RNA complexes, a feature interesting for the development of antiviral strategies based on double-stranded RNA targeting (Fei et al. 2009), together with an evaluation of the possibility to form supramolecular networks in aqueous solution based on the heteroaromatic derivative, which could prove an useful strategy for future biotechnological applications.

Materials and methods

Chemicals, apparatus and general methods

CH_3CN, K_2CO_3, HCl and EtOH were obtained from "Aldrich" (USA). The cationic exchanger Ku-2x8 was purchased from Reachem. All used solvents were freshly distilled. Poly rA was provided by Fluka and poly rU by Sigma.

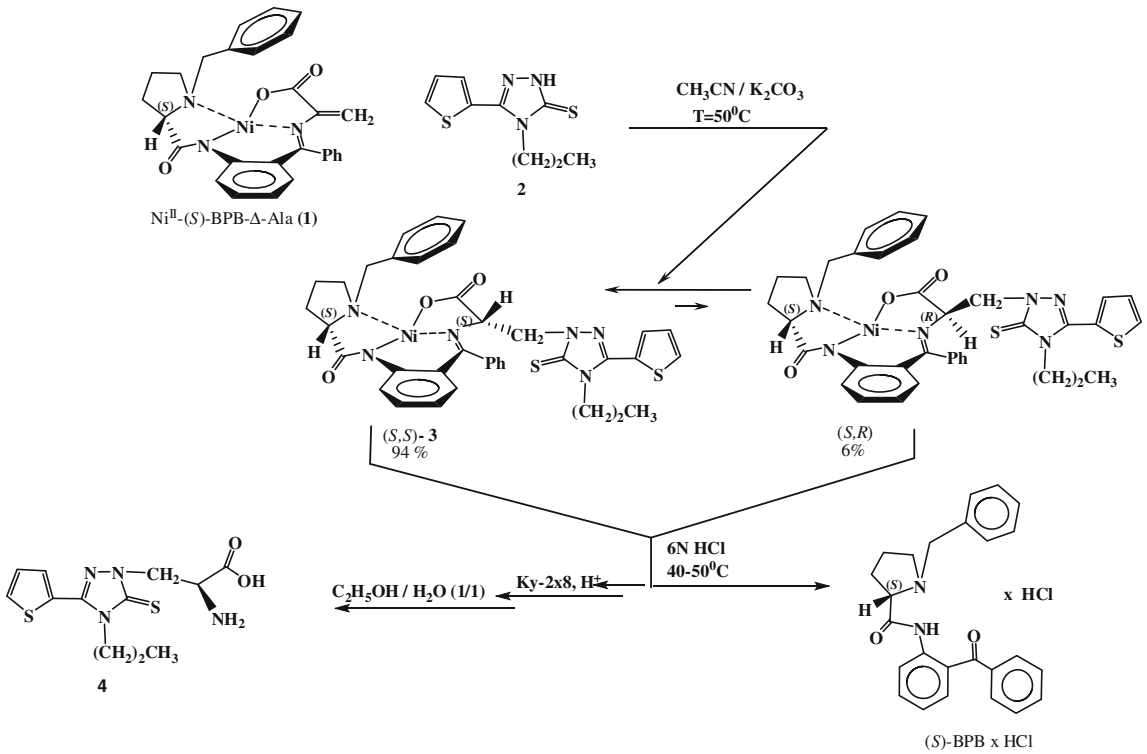
^1H NMR spectra were recorded on a "Mercury-300 Varian" (300 MHz) in d_6-DMSO/CCl_4:1/3 (unless otherwise indicated). Enantiomeric purity of amino acid was determined by HPLC on the chiral phase Diaspher-110-Chirasel-E-PA 6.0 mkm 4.0 × 250 mm with 20 %–MeOH–80 % 0.1 M $NaH_2PO_4 \cdot 2H_20$ used as an eluent. The optical rotation was measured on a "Perkin Elmer-341" polarimeter. Centrifugations were performed on a Z200 A Hermle centrifuge. The product was 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, using a Phenomenex Proteo (4 μm, 4.6 × 150 mm) column. Gradient elution was performed using increasing amounts of acetonitrile (0.05 % TFA, B) in water (0.05 % TFA, A), with a linear gradient from 2 % (for 5 min)−30 % B in A over 10 min (t_R = 13.4 min), monitoring at 260 nm, with a flow rate of 0.8 ml/min.

UV measurements were recorded on a UV–Vis Jasco model V-530 spectrophotometer equipped with a Peltier ETC-505T temperature controller by using Hellma quartz Suprasil cells, with a light path of 1 cm and a Hellma Tandem quartz cell 2 × 0.4375 cm. All the UV melting curves reported in this work were performed on samples annealed in the following way: heating at 85 °C for 10 min following by slow cooling to 15 °C. All UV melting and cooling curves were recorded by monitoring the absorbance at 258 nm (data pitch, 10 s) upon increasing the temperature from 15 to 75 °C at 1 °C/min thermal rate.

Circular dichroism (CD) spectra were obtained on a Jasco J-715 spectropolarimeter, equipped with a Peltier PTC-423S/15 temperature controller, using a Hellma Tandem quartz cell 2 × 0.4375 cm. The parameters used for CD measurements were: data pitch = 1 nm, scanning speed = 50 nm/min, response: 4 s, band width = 2 nm, accumulations = 5. All UV and CD spectra were obtained at 10 °C. Melting temperature values were determined by the first derivative method.

Dynamic laser light scattering studies were conducted on a Zetasizer Nano ZS (Malvern Instruments ltd). A stock solution of the nucleoamino acid was filtered through a 0.2 μm Millex syringe driven filter unit (Millipore, Bedford, MA). After dilution, samples containing the nucleoamino acid were prepared in 10 mM sodium phosphate buffer, pH 7.5. All measurements were performed in triplicates for a 2 min acquisition time. The hydrodynamic diameter of the scattering molecules was derived using the Malvern software from the diffusion coefficient by the Einstein–Stokes equation.

The serum stability assay was performed following a procedure already reported by us in literature (Roviello et al. 2009). After incubation, the withdrawn samples were analyzed by HPLC on a Phenomenex Juppiter C18 300 Å (5 μm, 4.6 × 250 mm) column using a linear gradient from 2 % (for 5 min)–30 % B in A over 10 min (t_R = 14.0 min).



Scheme 1 Synthesis of the amino acid **4**

Synthesis of complex **3**

The chiral Ni^{II} complex of Schiff's base of dehydroalanine (Δ-Ala) with chiral auxiliary (*S*)-2-*N*-(*N*'-benzylprolyl)aminobenzo-phenone (Ni^{II}-(*S*)-BPB-Δ-Ala) was synthesized according to the procedure reported previously (Saghyan et al. 2003). The addition of heterocyclic nucleophile **2** (0.03 mol, Scheme 1) to the chiral dehydroalanine complex **1** (0.015) was carried out in MeCN (20 ml) in the presence of K_2CO_3 (0.045 mol) at 50 °C. The progress of the reaction was monitored by TLC on SiO_2. The reaction afforded a mixture of the diastereomeric complexes with a large excess of the diastereomer with (*S*)-configuration of the generated new chiral center. The diastereomeric mixture of the complexes was crystallized from methanol.

Complex **3**. Yield 63 %: Mp 118−120 °C: $[\alpha]_D^{20}$ = $+1{,}130.00^0$ (C = 0.06, MeOH). Found (%): C 60.34; H 4.77; N 11.51: Calc for $C_{37}H_{36}N_6NiO_3S_2$: (%): C 60.43; H 4.90; N 11.43: ^{1}H NMR ($CDCl_3$, δ, p.p.m, J/Hz): 0.99 (3H, t, ^3J = 7.4, CH_3); 1.83 (2H, m, N-$CH_2CH_2CH_3$); 2.04 (1H, m, δ-H_a Pro); 2.04 (1H, m, γ-H_a Pro); 2.45 (1H, m, β-H_a Pro); 2.76 (1H, m, β-H_a Pro); 3.37 (1H, dd, ^3J = 10.6, ^3J = 6.1, α-H Pro); 3.55 (1H, m, δ-H_b Pro); 3.57 (1H, d, ^2J = 12.6, CH_2Ph); 3.59 (1H, m, γ-H_b Pro); 4.14 (2H, *m*, N-$CH_2CH_2CH_3$); 4.39 (1H, d, ^2J = 12.6, CH_2Ph); 4.42 (1H, t, ^3J = 6.4, CH_2CH); 4.83 (1H, dd, ^2J = 13.6, ^3J = 6.4, $CHCH_2$); 4.88 (1H, dd, ^2J = 13.6, ^3J = 6.4, $CHCH_2$); 6.58 (1H, ddd, ^3J = 8.3, ^4J = 2.2, H-3, C_6H_4); 6.61 (1H, ddd, ^3J = 8.3, ^3J = 6.5, ^4J = 1.0, H-4, C_6H_4); 7.05 (1H, t, d, ^3J = 7.7, ^4J = 1.5, H-2, C_6H_5); 7.09−7.14 (2H, m, H-Arom); 7.17 (1H, tt, ^3J = 7.8, ^4J = 1.5, H-4, C_6H_5); 7.24−7.36 (4H, m, H-Arom); 7.38−7.56 (4H, m, H-Arom); 8.00 (2H, m, H-2, 6, C_6H_5); 8.28 (1H, d, ^3J = 8.7, H-6, C_6H_4,).

Isolation of the (*S*)-β-[3-(thiophen-2-yl)-4-propyl-5-thioxo-1,2,4-triazol-1-yl)-α-alanine (4)

The diastereomeric mixture of the complex was decomposed by an excess of HCl in aqueous methanol, and the amino acid was isolated using Ku-2x8 cationic exchanger (Saghyan et al. 2012, 2013). Amino acid **4** was crystallized from water ethanol (1:1). The optical purity of the synthesized amino acid was determined by chiral HPLC. The chiral auxiliary (*S*)-BPB was recovered in a yield of >95 % with retention of optical purity and could be reused. (*S*)-β-[3-(thiophen-2-yl)-4-propyl-5-thioxo -1,2,4-triazol-1-yl)-α-alanine (4): Yield 45 %: ee = 91, 78 %: Mp 256−258 °C. Found (%): C 46,15; H 5,38; N 19.61: Calc for $C_{12}H_{16}N_4O_2S_2$ (%): C 41.81; H 5.22; N 19.51: $[\alpha]_D^{20} = -5.45^0$ (C=0.348, C_2H_5OH/H_2O = 1/1): ^{1}H NMR (DMSO, δ, ppm, J/Hz): 0.96 (3H, t, ^3J = 7.4, CH_3); 1.76 (2H, m, $CH_2CH_2CH_3$); 4.12 (2H, m, $CH_2CH_2CH_3$); 4.44 (1H, dd, ^3J = 4.9, ^3J = 8.0, CH); 4.54 (1H, dd, ^2J = 14.1,

^{3}J = 8.0, $CHCH_2$); 4.76 (1H, dd, ^{2}J = 14.1, ^{3}J = 4.9, $CHCH_2$); 7.21 (1H, dd, ^{3}J = 5.1, ^{3}J = 3.7, H-4 Thiophen); 7.58 (1H, dd, ^{3}J = 3.7, ^{4}J = 1.1, H-3 Thiophen); 7.70 (1H, dd, ^{3}J = 5.1, ^{4}J = 1.1, H-2 Thiophen). ^{13}C-NMR (DMSO/CF_3COOD 1/3): 8.7 (CH_3); 19.8 (CH_2); 46.2 (NCH_2); 46.3 (NCH_2); 50.5 (CH); 123.1 (=C); 127.2 (=CH); 129.5 (=CH); 129.5 (=CH); 146.2 (=C); 165.9 and 167.6 (C=O and C=S). ESI–MS *m/z*: 312.72 (found), 313.42 (required for M+H$^+$). The amino acid was treated with ethanol (with a concentration of 1.3 mg/mL) to be used in the various assays described.

Results and discussion

In order to perform the asymmetric synthesis of the heteroaromatic amino acid object of the present study, a chiral Ni^{II} complex of Schiff's base of dehydroalanine (Δ-Ala) with the chiral auxiliary (*S*)-2-N-(N'-benzylprolyl)aminobenzo-phenone (Ni^{II}-(*S*)-BPB-Δ-Ala) was synthesized according to a procedure reported previously (Saghyan et al. 2003). Subsequently, heterocyclic nucleophil **2** (Scheme 1) was added to the chiral dehydroalanine complex in the presence of K_2CO_3 monitoring the reaction progression by TLC. This procedure led to the formation of a mixture of diastereomeric complexes with a large excess of the diastereomer with (*S*)-configuration of the new chiral center generated. The diastereomeric mixture of complexes was crystallized from methanol and after treatment with an excess of HCl in aqueous methanol, the amino acid was isolated using a cationic exchanger. Amino acid **4** was, thus, obtained after crystallization from water:ethanol (1:1) in 45 % yield and with a high optical purity (ee = 91, 78 %), as determined by chiral HPLC.

The artificial amino acid was fully characterized by NMR and MS techniques. In particular, LC-ESIMS analysis (positive ions, Fig. 2) showed only one HPLC peak corresponding to a *m/z* value of 312.72 (313.42 *m/z* was required for $C_{12}H_{16}N_4O_2S_2$ + H$^+$). The corresponding UV spectrum in CH_3CN/H_2O, 3:7 (0.05 % TFA) showed three bands at 215, 261 and 297 nm.

Subsequently, a biological assay was conducted on the heteroaromatic compound to evaluate its resistance to degradation *in sero*. More in particular, the enzymatic resistance of the amino acid was investigated by incubating the compound in 99 % fresh human serum at 37 °C (the concentration of the amino acid was 5 μM) and analyzing by HPLC samples withdrawn from the reaction mixture at

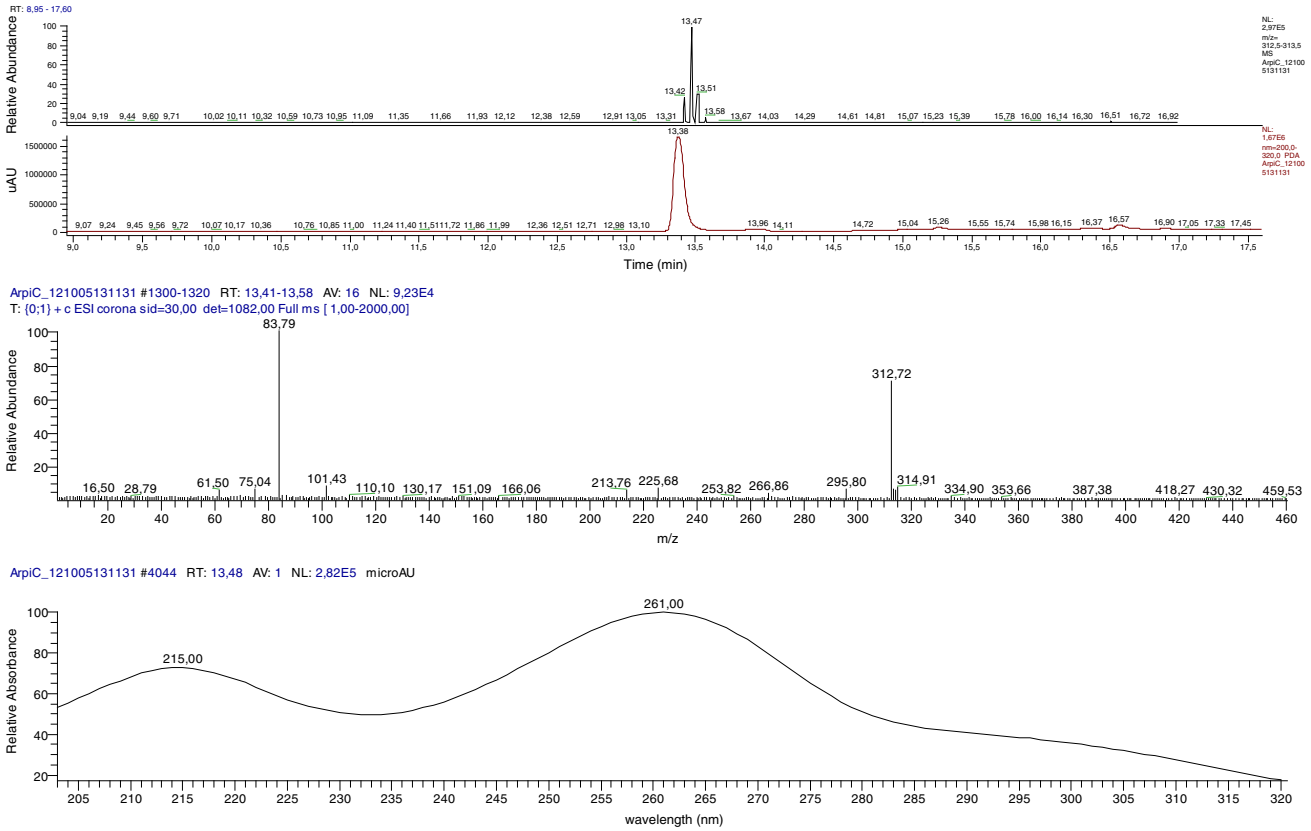


Fig. 2 LC-ESIMS of the amino acid **4**

various times: even after 24 h, the artificial compound was still present suggesting a good stability of the molecule (Fig. 3).

UV and CD spectroscopies were also employed in order to assess the ability of the molecule to interact with RNA–RNA complexes, which is an important feature in the field of the discovery of innovative antiviral drugs, as modulating of complementary RNA–RNA interaction by small molecules could affect the survival of several viruses. More in detail, a RNA–RNA complex was first formed using two complementary strands, i.e. poly rU and poly rA. Such RNA molecules were solved in aqueous solution under controlled pH conditions (7.5 nmol in base of each RNA were solved in 0.8 mL of 10 mM phoshate buffer solution).

The two RNA solutions were placed separately in the two chambers of which the tandem cell is composed and both UV and CD "sum" spectra were recorded (black lines, Fig. 4). After manual rotation of the cell and the consequent mixing of the two solutions, complex spectra were recorded in the same experimental conditions (red lines, Fig. 4). Changes in UV and CD spectra confirmed the formation of the poly rU/poly rA complex as expected. After thermal denaturation and subsequent correct annealing, the heating curve of the complex showed a T_m of about 42 °C as revealed by UV thermal experiment (Fig. 5).

Furthermore, analogue UV and CD experiments were conducted by introducing in one of the chambers of the tandem cell the poly rU/poly rA complex (2.35 μM in base for each RNA, 10 mM phosphate buffer, pH = 7.5) and in the other one a sample of the artificial amino acid (9.4 μM, 10 mM phosphate buffer, pH = 7.5).

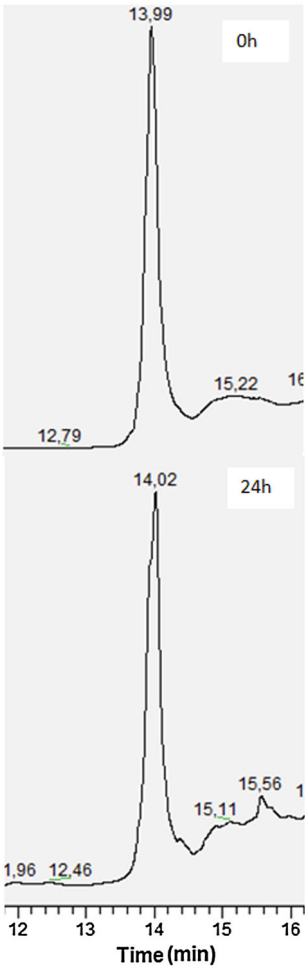


Fig. 3 Serum stability assay on the heteroaromatic amino acid (t_R = 14.0 min) incubated with human serum. After the indicated incubation time, samples were analyzed by C18 RP-HPLC. Method for RP-HPLC analysis: 2−30 % MeCN (0.1 % TFA) in H_2O (0.1 % TFA) over 10 min

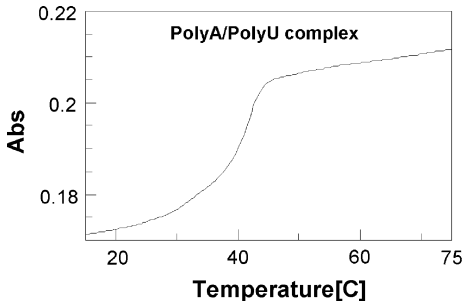


Fig. 5 UV melting curve (absorbance recorded at 258 nm) for the poly rU/poly rA complex (4.7 μM in base for each RNA in 10 mM sodium phosphate buffer solution, pH 7.5). Path length, 1 cm; temperature scan rate, 1 °C/min

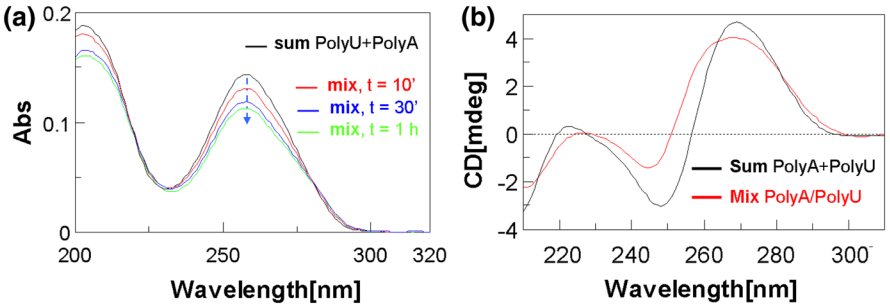


Fig. 4 UV (**a**) and CD (**b**) spectra related to the kinetic formation of the poly rU/poly rA complex in 10 mM phoshate buffer, pH 7.5 (T = 10 °C), in a tandem cell

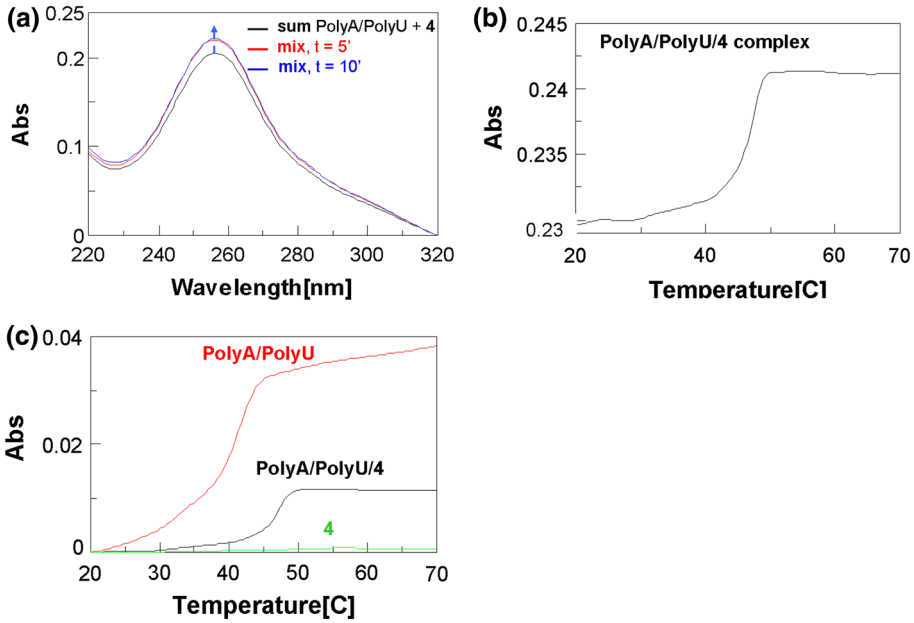


Fig. 6 **a** UV sum (*black*) and complex (*red* and *blue*) spectra relative to the formation of the amino acid/poly rU/poly rA complex in 10 mM sodium phosphate buffer, pH 7.5. **b** UV melting experiment (absorbance recorded at 258 nm) performed on the amino acid/poly rU/poly rA (ternary) complex (2.35 μM in base of each RNA and 9.4 μM of **4** in 10 mM phosphate buffer, pH 7.5). Path length, 1 cm; temperature scan rate, 1 °C/min. **c** Comparison of the UV heating curves relative to: poly rU/poly rA complex (*red line*), ternary complex (*black line*) and amino acid **4** alone (*green line*), assuming a zero value for the absorbance at 20 °C for all the curves (color figure online)

Sum (black) and complex (red and blue) UV spectra were obtained and also in this case a change in the spectral profile was detectable suggesting an interaction of the heteroaromatic amino acid with the RNA complex (Fig. 6a). The UV study of the thermal stability of the ternary complex (Fig. 6b) revealed a transition at about 47 °C. Moreover, the difference in the UV heating curves of (1) the RNA complex (red line, Fig. 6c) (2) the ternary system (black line) and (3) the amino acid **4** alone (green line), in the same experimental conditions, further confirmed the formation of a ternary complex involving the artificial amino acid and the nucleic acid. The change in absorbance (ΔAbs) observed upon heating for the ternary complex was lower than that found for the poly rU/poly rA complex, probably due to a partial destabilizing effect of **4** on the RNA complex.

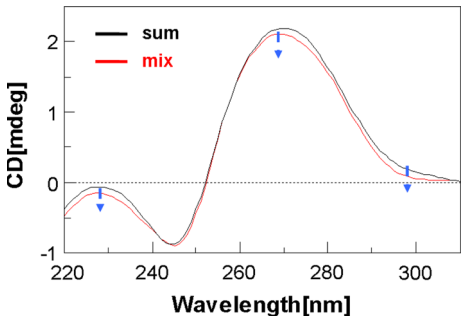


Fig. 7 CD sum (*black*) and complex (*red*) spectra relative to the formation of the amino acid/poly rU/poly rA complex in 10 mM sodium phosphate buffer, pH 7.5 ($T = 10$ °C), in a Tandem cell (reservoir 1: 2.35 μM in base of each RNA; reservoir 2: 9.4 μM of amino acid) (color figure online)

Also a CD experiment performed in the same experimental conditions already employed in the UV assay, confirmed the formation of a complex, in which however **4** does not significantly alter the global conformation of the nucleic acid, since only a slight variation in the CD spectrum at about 295, 270 and 230 nm was detectable upon mixing of the amino acid and RNA solutions (Fig. 7). Interestingly, CD binding experiments were also performed with the RNA single strands (poly rU and poly rA), but no binding was detected in both cases.

Finally, dynamic laser light scattering studies (Fig. 8) were conducted on the artificial compound which evidenced the formation of aggregates corresponding to hydrodynamic diameters higher than 200 nm. This result suggests the formation of non-covalent polymeric networks based on multiple amino acid units held together by weak interactions.

Conclusions

In the present work we reported a convenient asymmetric synthesis of an artificial amino acid and its NMR and ESI–MS characterization, as well as the study of some biologically relevant properties of the new compound. More in detail, the heteroaromatic compound proved to be resistant to enzymatic degradation, as well as able to form supramolecular networks (characterized by hydrodynamic diameters higher than 200 nm) constituted by multiple units of the heteroaromatic compound held together by weak interactions, feature that could be useful in drug delivery strategies. Moreover, the ability of the synthetic amino acid to interact with RNA complexes was

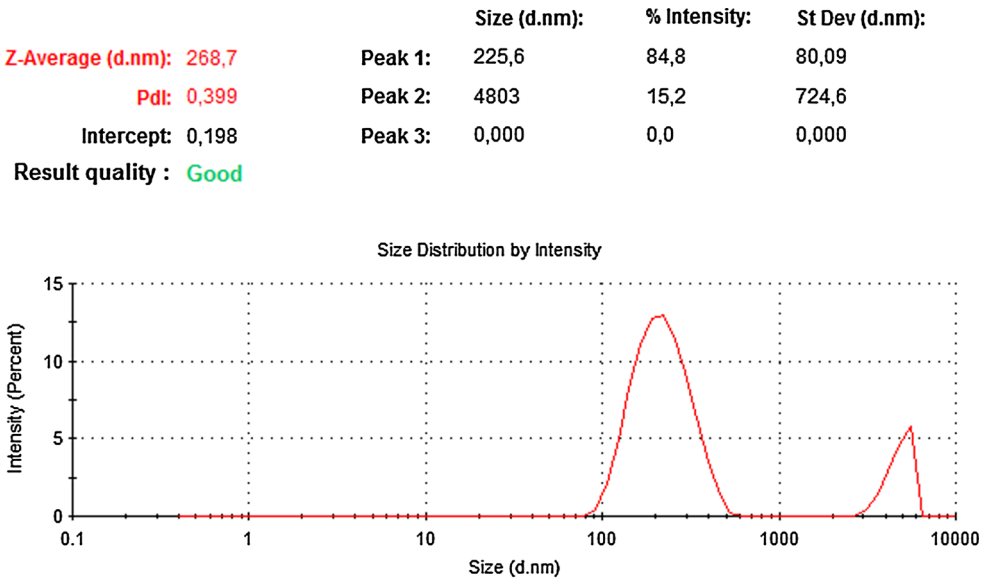


Fig. 8 Aggregation study by dynamic laser light scattering performed on a sample of artificial amino acid (8 μM in H_2O-0.2 % EtOH) at 25 °C

ascertained by studying its molecular recognition of a poly rU/poly rA complex and is of interest for potential antiviral strategies, since several processes mediated by RNA/RNA recognition are fundamental for the survival of different viruses. In this regard, in vitro cellular assays will be object of our future research in collaboration with specialized research groups in order to verify the potential biological activity of the new amino acid.

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

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