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ORIGINAL ARTICLE

Synthesis of a novel Fmoc-protected nucleoaminoacid for the solid phase assembly of 4-piperidyl glycine/L-arginine-containing nucleopeptides and preliminary RNA interaction studies

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Abstract In this work, we report the synthesis of a novel Fmoc-protected nucleoaminoacid, based on 4-piperidinyl glycine, carrying the DNA nucleobase on the secondary amino group, suitable for the solid-phase synthesis of nucleopeptides. After ESI–MS and NMR characterization this building block was used for the assembly of a thymine-functionalized tetrapeptide, composed of 4-piperidinyl glycine and L-arginine moieties alternated in the backbone. The ability to interact with RNA and the efficiency in interfering with the reverse transcription of eukaryotic mRNA of the novel nucleo-tetrapeptide found in this study are in favour of the employment of chiral nucleopeptides based on alternate 4-piperidinyl glycine/L-arginine backbone in biomedicine.

Keywords Nucleopeptide · RNA · CD · RT–PCR

Abbreviations

Ac_2O	Acetic anhydride
Boc	*tert*-Butoxycarbonyl
DCM	Dichloromethane
DIEA	*N,N*-Diisopropylethylamine
DMF	*N,N*-Dimethylformamide
DMSO	Dimethylsulfoxide
Fmoc	9-Fluorenylmethoxycarbonyl
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HATU	*O*-(7-Azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate
MBHA	4-Methylbenzhydrylamine
NMP	4-Methylpyrrolidone
Pbf	2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl
TAE	Tris-acetate-EDTA
TCH_2COOH	Thymin-1-yl acetic acid
TFA	Trifluoroacetic acid
TIS	Triisopropyl silane

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Introduction

In the last decades, several investigations employing antigene or antisense strategies for modifying replication, transcription and translation have shown that the ribose phosphodiester linkage can be replaced by various modifications (Bell and Micklefield 2009). For example, the extensive work of Nielsen et al. (1991) on peptide nucleic acids (PNAs) showed that oligonucleotide analogs with a pseudopeptide-like backbone can interact with natural nucleic acids by forming complexes of high thermal stability. Since then, many modified PNAs have been reported in literature including positively charged PNAs with remarkable cell-permeability properties (Katritzky and Narindoshvili 2008; Dragulescu-Andrasi et al. 2006), piperidine-based PNAs synthesized starting from the

naturally occurring 4-hydroxy-L-proline (Lonkar and Kumar 2004), as well as PNAs based on the six membered *trans*-5-aminopipecolic acid, which showed interesting binding characteristics towards DNA and RNA (Lonkar and Kumar 2005).

Nevertheless, besides the PNAs, several attempts to use real peptides as alternative oligonucleotide linkages have also been reported, allowing for obtaining chiral nucleopeptides with interesting properties (Geotti-Bianchini et al. 2008; Roviello et al. 2010). As a general rule, in a nucleopeptide backbone isomorphous to DNA and RNA, six bonds of the sugar–phosphate backbone are correlated, in terms of their length, to two amino acids. Therefore, the repeating unit of a potential nucleic acid-binding nucleopeptide is built by an amino acid pair containing a nucleobase-substituted and a proteinogenic amino acid (Diederichsen 1996).

Taking into account all these considerations, together with the importance of the piperidine-containing PNAs (Lonkar and Kumar 2004), we designed and realized a novel analog of thymine dinucleotide characterized by a real peptide backbone comprising both 4-piperidinyl glycine and L-arginine moieties alternated in the sequence. In this oligomer, the atoms bringing the nucleobases were separated by the same number of bonds (i.e. 6) found in PNA while the DNA bases were anchored to the secondary amino groups of 4-piperidinyl glycine units. L-Arginines were introduced as spacers between the nucleobase-carrying amino acids in order (1) to improve the water-solubility of the resulting nucleopeptide, (2) to promote the hybridization of the positively charged analog to the negatively charged nucleic acids and, not less importantly, (3) to enhance its cell permeability. The ability to interact with RNA as well as some biological properties of the novel alternate 4-piperidinyl glycine/L-arginine nucleopeptide were studied and described in the present work.

Materials and methods

Chemicals

Boc-L-Arg(Pbf)-OH and HATU 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. PolyA, TFA were from Fluka. Rink-MBHA-amide resin was purchased from Advanced Biotech Italia. DCM, DIPEA and TFA (for HPLC) were from Romil. Deuterated DMSO, Fmoc-*N*-(1-Boc-4-piperidyl) glycine, TCH_2COOH and TIS were from Sigma–Aldrich.

Apparatus

^1H NMR and ^{13}C NMR spectra were recorded at 25°C on Varian unity 400 MHz spectrometers. Chemical shifts (δ) are given in parts per million (ppm). Proton chemical shifts were referenced to residual CHD_2SOCD_3 ($\delta = 2.49$, quin) signals. ^{13}C NMR chemical shifts were referenced to the solvent (CD_3SOCD_3 $\delta = 39.5$, sept). Crude samples containing nucleopeptides were centrifuged for 4 min at 4,000 rpm (Z 200 A, Hermle). Products were analyzed by LC–MS performed on an MSQ mass spectrometer (ThermoElectron, Milan, Italy) equipped with an ESI source operating at 3 kV needle voltage and 320°C, and with a complete Surveyor HPLC system comprising an MS pump, an autosampler, and a PDA detector using a Phenomenex Jupiter C18 300 Å (5 µm, 4.6 × 150 mm) column. Gradient elution was performed (monitoring at 260 nm) by building a gradient starting with buffer A (0.05% TFA in water) and applying buffer B (0.05% TFA in acetonitrile) with a flow rate of 0.8 ml/min.

Semi-preparative purifications were performed by RP-HPLC on a Hewlett Packard/Agilent 1100 series, equipped with a diode array detector, 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 a gradient starting with buffer A (0.1% TFA in water) and applying buffer B (0.1% TFA in acetonitrile) with a flow rate of 4 ml/min. Samples containing nucleopeptides (crude or purified) were lyophilized in a FD4 Freeze Dryer (Heto Lab Equipment) for 16 h.

Circular dichroism (CD) spectra were obtained on a Jasco J-810 spectropolarimeter, while 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 and a Hellma Tandem quartz cell 2 × 0.4375 cm (Rocchi et al. 1972; Krzyzanowska et al. 1998).

Synthesis of the Fmoc-*N*-[1′-(thymin-1-ylacetyl) -4′-piperidyl]glycine **(2)**

Fmoc-N-(4-piperidyl)glycine, ***1*** *(Scheme 1)*

Commercial Fmoc/Boc-protected 4-piperidinyl glycine **1** (72 mg, 0.15 mmol) was treated with a solution of TFA/DCM 1:1 (2 ml) at 25°C. After 1 h stirring the solvent was removed under vacuum. The obtained crude was treated with cold diethyl ether and after centrifugation a white precipitate was recovered (57 mg of **1**, 0.15 mmol, 100% yield). This precipitate contained the desired product substantially pure. δ_H (400 MHz, DMSO-d_6) 8.73–8.325 (2H, m, COOH, NH), 7.93–7.34 (8H, m, aromatic

CH Fmoc), 4.55–3.68 (6H, m, FmocCH-CH_2, FmocCH-CH_2, NCH_2COOH, NCHCH_2), 3.39–2.66 (4H, m, N(CH_2CH_2)$_2$), 1.99–1.66 [4H, m, NCH(CH_2CH_2)$_2$]; δ_C (100 MHz, DMSO-d_6) 175.3, 158.8, 147.6, 144.6, 131.6, 131.0, 129.0, 123.9, 71.1, 55.4, 50.6, 48.8, 46.9, 29.8. ESI–MS *m/z*: 381.76 (found), 381.46 (expected for $[C_{22}H_{24}N_2O_4 + H]^+$); 403.44 (found), 403.44 (expected for $[C_{22}H_{24}N_2O_4 + Na]^+$); 761.49 (found), 761.90 (expected for $[2(C_{22}H_{24}N_2O_4) + H]^+$); 783.88 (found), 783.88 (expected for $[2(C_{22}H_{24}N_2O_4) + Na]^+$).

Fmoc-N-[1′-(thymin-1-ylacetyl)-4′-piperidyl]glycine, ***2*** *(Scheme 1)*

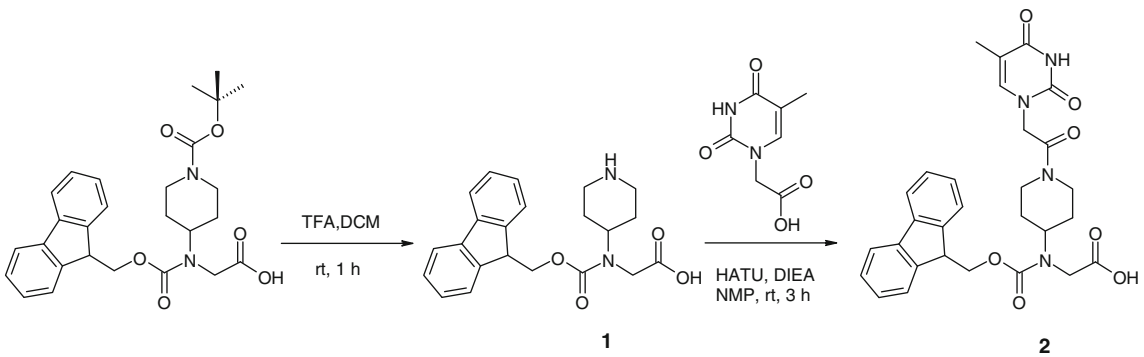
Product **1** (57 mg, 0.15 mmol) was dissolved in dry NMP (500 µl), treated with DIEA (2.5 equiv., 64 µl, 0.38 mmol) and reacted with TCH_2COOH (2.8 equiv., 77 mg, 0.42 mmol) which was previously preactivated with HATU (2.4 eq, 134 mg, 0.35 mmol) and DIEA (4.1 eq, 105 µl, 0.62 mmol) in NMP (500 µl) for 2 min. After 3 h the reaction was quenched by adding 700 ml water and after freezing the sample was lyophilized. The crude material was treated with water (50 ml) and after sonication the aqueous solution was removed from the white material which prevalently contained one product (**2**, 77 mg, 0.14 mmol, 94% yield). δ_H (400 MHz, DMSO-d_6) 12.60 (1H, bs, COOH), 11.32 (1H, s, NH thymine), 7.98–7.36 (9H, m, aromatic CH Fmoc, CH thymine), 4.60 (2H, d, FmocCH-CH_2), 4.41–4.19 (5H, m, CH_2 acetyl linker, FmocCH-CH_2 and NCH_2COOH), 3.51–3.42 (5H, m, NCHCH_2 and N(CH_2CH_2)$_2$), 1.80 (3H, s, CH_3 thymine), 1.78–1.62 [4H, m, NCH(CH_2CH_2)$_2$]; δ_C (100 MHz, DMSO-d_6) 175.3, 168.5, 159.0, 155.1, 147.7, 146.3, 144.7, 132.9, 131.5, 131.1, 128.7, 124.0, 112.0, 70.5, 57.8, 52.1, 50.7, 47.5, 45.3, 32.7, 15.8. ESI–MS *m/z*: 547.72 (found), 547.60 (expected for $[C_{29}H_{30}N_4O_7 + H]^+$); 569.76 (found), 569.57 (expected for $[C_{29}H_{30}N_4O_7 + Na]^+$).

Solid phase synthesis of oligomer **3**

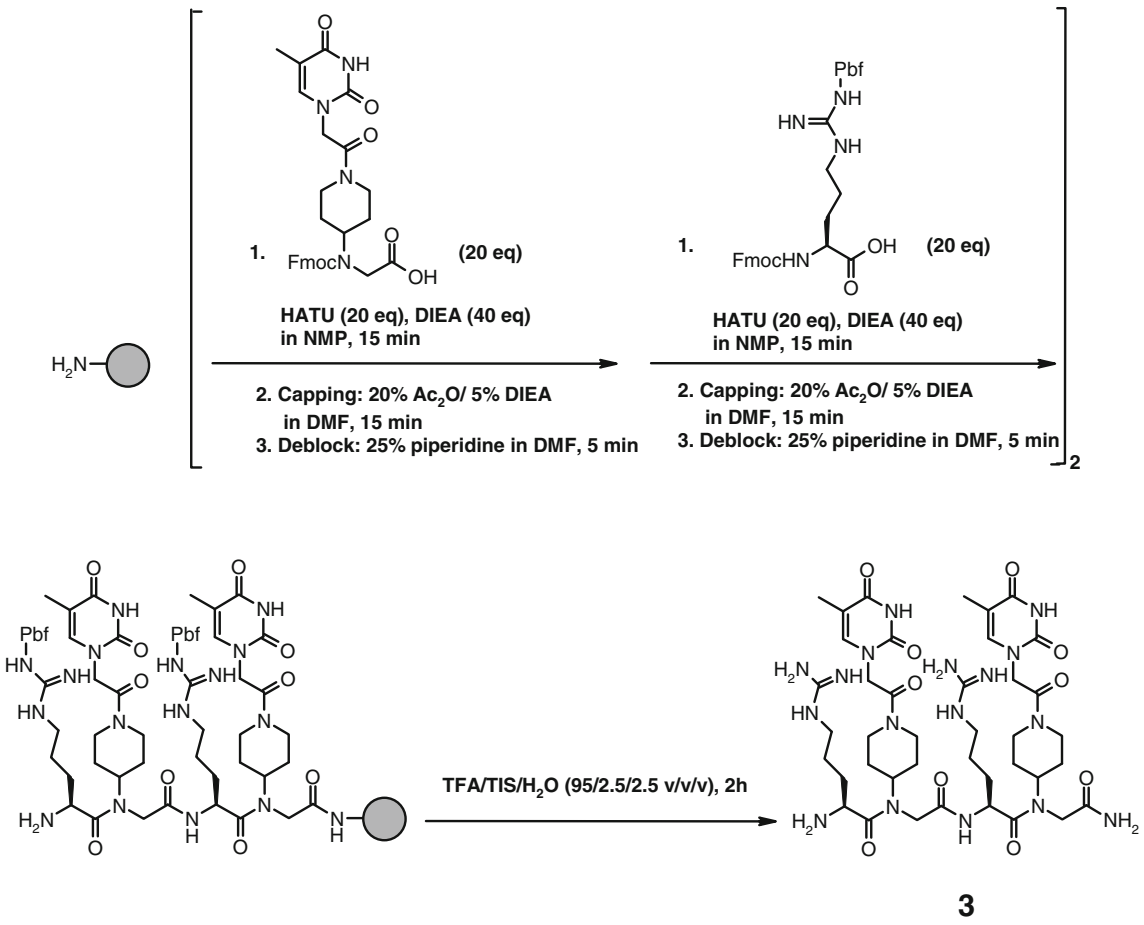
Oligomer **3** was assembled on Rink-amide MBHA resin (0.54 mmol/g, 11.7 mg, 6.3 µmol) using the synthetic strategy described in Scheme 2. More particularly, monomer **2** or Fmoc-L-Arg(Pbf)-OH (126 µmol, 20 equiv.) previously pre-activated with HATU (126 µmol, 20 eq) and DIEA (252 µmol, 40 equiv.) in NMP for 2 min, were added to the NH_2-resin and the mixture was reacted for 30 min under stirring. Each condensation step was followed by capping performed with 20% Ac_2O/5% DIEA in DMF for 15 min, while, Fmoc deprotection was achieved with piperidine (25%) in DMF for 5 min. The yields for each coupling step were around 60% (as estimated by UV Fmoc test). The nucleopeptide was then cleaved from the solid support by treatment with TFA:TIS:H_2O (95:2.5:2.5 v/v/v) over 2 h and recovered by precipitation with cold diethyl ether, centrifugation and lyophilization. The oligomer **3** was purified by semipreparative HPLC using a linear gradient of 5% (for 5 min) to 20% B in A over 20 min: $t_R = 25.3$ min; UV quantification of the purified product gave 630 nmol of **3**; ESI–MS (Fig. S2) *m/z*: 943.84 (found), 943.06 (expected for $[C_{40}H_{63}N_{17}O_{10} + H]^+$); 472.46 (found), 472.03 (expected for $[C_{40}H_{63}N_{17}O_{10} + 2H]^{++}$).

UV and CD studies

The purified oligomer **3** was dissolved in milliQ water and quantified by UV measurements ($T = 80$°C, absorbance value at $\lambda = 260$ nm). The epsilon value used for the quantification of the oligomer **3** (17.2 mM^{-1}) was calculated using the molar extinction coefficient of thymine PNA monomer (8.6 mM^{-1}). CD spectra were recorded with the following parameters: scan speed 50 nm/min, data pitch 2 nm, band width 2 nm, response 4 s, 5 accumulations.



Scheme 1 Synthesis of monomer **2**



Scheme 2 Synthesis of oligomer **3**

Cell culture

HeLa cells (ATCC, USA) were grown in RPMI1640 medium supplemented with 10% fetal bovin serum (FBS), 1% glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA), at 37°C and 5% CO_2.

RT–PCR

Total RNA was extracted from the cellular lysate using Tri-reagentTM (Sigma–Aldrich, St. Louis, MO) according to the manufacturer's instructions. RT was performed using 0.5 μg of total RNA, 200 U of MMLV Reverse Transcriptase RNase H (Finnzymes, Espoo, Finland), 250 ng of dT15 primer (Roche, Switzerland) in the presence of 1 or 10 μg of oligomer **3**. A preincubation mixture containing RNA and **3** was performed for 10 min. Reaction temperature was set at 22°C for 1 h. After RT, PCR assay of GAPDH and β-actin transcripts was carried out using the following primers: forward primer 5′-ATGGGGAAGGTGAAGGTC-3′, reverse primer 5′-GTCATGGATGACCTTGGC-3′ and forward primer 5′- TGAGACCTTCAACACCCC-3′, reverse primer 5′-CAGGAAGGAAGGCTGGAA-3′ (purchased by Sigma-Genosys Ltd), respectively. The PCR protocol was as follows: 5 min at 95°C followed by 25 cycles of 1 min at 95°C, 1 min at 58°C and 1 min at 72°C. PCR products were then analyzed on 1% agarose gel in TAE buffer and visualized by ethidium bromide staining. Gel images were captured by a ChemiDocTM XRS and analyzed by Quantity-One software (Biorad, Hercules, CA).

Results and discussion

The synthesis of the new 4-piperidinyl glycine-based thymine monomer (**2**), suitably protected for peptide solid phase synthesis (Fmoc chemistry), is reported in Scheme 1 and starts from the commercial Fmoc-*N*-(1-Boc-4-piperidyl)glycine. After removal of the Boc group, achieved by acid treatment, Fmoc-*N*-(4-piperidyl)glycine intermediate **1** was obtained in quantitative yield. Subsequently, compound **1** was reacted with thyminyl acetic acid using HATU/DIEA as a coupling system. After solvent removal

and HPLC purification Fmoc-protected nucleoaminoacid **2** was obtained in 94% yield. LC–ESI–MS (Fig. S1) characterization confirmed the identity of both compound **1** and monomer **2**.

Subsequently, a dithymine tetrapeptide **3** was synthesized in solid phase by using the monomer **2** and the commercial Fmoc-L-Arg(Pbf)-OH with HATU/DIEA in NMP as a coupling system (Scheme 2).

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–ESI–MS characterization confirmed the identity of the dinucleotide analog (Fig. S2). The resulted compound is well soluble in water and did not show any tendency to aggregate.

Furthermore, the structural characteristics of the 4-piperidinyl glycine-based nucleopeptide as well as its ability to interact with complementary RNA was investigated by CD spectroscopy. Firstly, the CD profile of the single strand in 10 mM, phosphate buffer pH 7.5 was analysed in order to evaluate any pre-organization of the molecule. By examining the CD behaviour of the molecule **3** in the range 190–240 nm at different temperatures (5, 10 and 25°C) no significant α-helical contribution was detected for the single strand (data not shown).

CD binding experiments on the dithymine nucleotetrapeptide with the complementary RNA (polyA) were then performed in a tandem cell recording the sum CD spectrum of the separated components and the mix CD spectrum, recorded after cell mixing, in order to evaluate its ability to interact with RNA, an important property in view of possible biomedical applications. More in detail, a 8 μM in nucleobase (T) solution of nucleopeptide **3** in 10 mM phosphate buffer pH 7.5 was introduced in one of the two tandem cell reservoirs and the CD spectrum was recorded (red line, Fig. 1). Subsequently, a 8 μM in nucleobase (A) solution of polyA RNA in 10 mM phosphate buffer pH 7.5 was introduced in the other reservoir and the "sum" CD spectrum was recorded (green line, Fig. 1). As expected, the CD of the single strand resulted weak as compared to the strong bands of the RNA evident in the "sum" CD spectrum.

After mixing the two solutions, a certain difference in the shape and intensity of the CD profile observed between the sum (green line, Fig. 1) and mix spectra (pink line, Fig. 1) revealed an interaction between the nucleopeptide and RNA.

Anyway, a more evident variation in CD signal was observed when a further equivalent in thymine of **3** was added to form a 2:1 = T:A complex (blue line, Fig. 1). Adding more than two equivalents of alternate nucleopeptide, we did not find any substantial CD variation according to a 2:1 stoichiometry required for a (nucleopeptide)$_2$/RNA complex formation (Fig. 1).

Biological properties

The biological activity of the alternate nucleopeptide was determined by interference of oligomer **3** with reverse-transcription (RT) reaction using total RNA from HeLa cells. In this experiment, RT was performed using a dT_{15} primer and different amounts of oligomer **2** (0, 1 and 10 μg). Then, cDNAs obtained were amplified by PCR using gene-specific primers for GAPDH and β-actin (constitutively expressed genes). We found that, in the presence

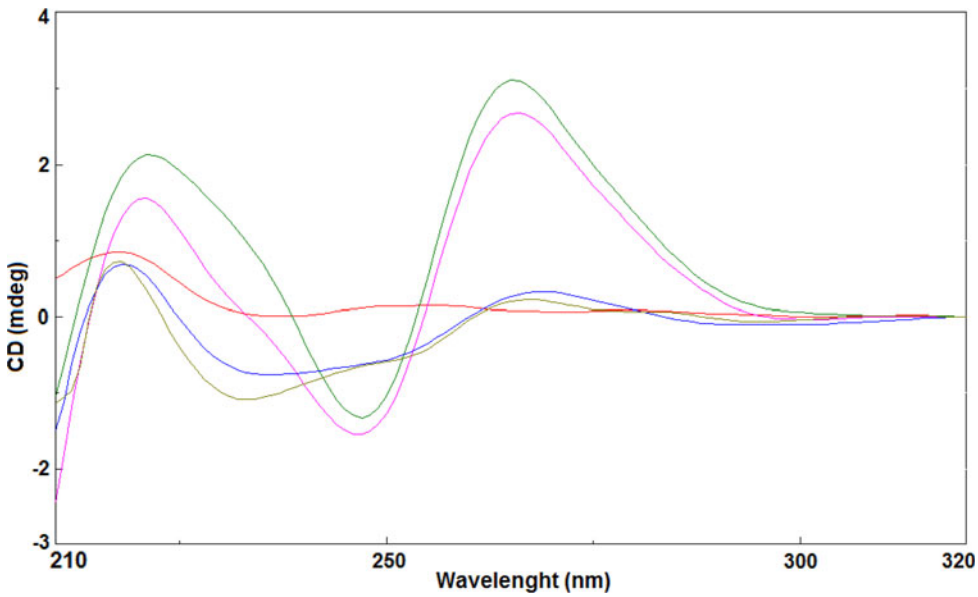


Fig. 1 CD binding study: 8 μM in nucleobase (T) solution of nucleopeptide **3** (*red*), 8 μM in T of nucleopeptide **3** + 8 μM in A of RNA (polyA) before (*green*) and after (*pink*) mixing, 16 μM in T of nucleopeptide **3** + 8 μM in A of polyA (*blue*), 24 μM in T of nucleopeptide **3** + 8 μM in A of polyA (*khaki*). All spectra were recorded at 10°C in 10 mM phosphate buffer pH 7.5 (color figure online)

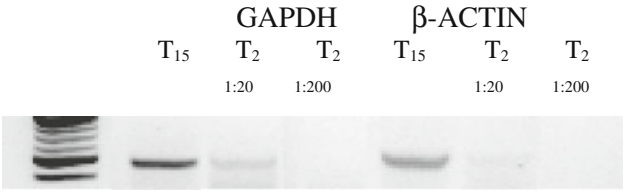


Fig. 2 Interference of oligomer **3** addition on RT–PCR reaction. A semiquantitative RT–PCR was performed using specific primers for GAPDH and *β*-actin in the presence of 1 μg (*lanes 3* and *6*) or 10 μg (*lanes 4* and *7*) of **3**. Molecular markers are in *lane 1*

of the new nucleopeptide **3**, the amplification of both genes was reduced. In particular, the reduction was quantified as 62% for GAPDH gene (lanes 4, Fig. 2) and 65% for *β*-actin gene (lanes 7 and 8, Fig. 2) in the presence of 1 μg of oligomer **3** (or 1:20 primer/nucleopeptide molar excess) when compared to PCR amplification in the absence of oligomer **3** (lanes 3 and 6, Fig. 2). The genes amplification was completely suppressed in the presence of 10 μg of **3** (or a 1:200 molar excess).

Conclusions

Here we reported the synthesis, purification and characterization of a novel Fmoc-protected nucleoaminoacid based on 4-piperidyl glycine, suitable for the solid phase synthesis and its oligomerization to the alternate nucleopeptide **3** with two thymine monomers and two L-arginine units using a solid phase synthetic route.

The novel nucleopeptide was purified by HPLC and characterized by LC–ESI–MS. Binding experiments, performed by CD spectroscopy showed that this peptide-like analog of thymine dinucleotide, which resulted well soluble in water and did not present any tendency to self-aggregate, was able to interact with a complementary RNA. Furthermore, preliminary biological assays showed that the novel analog was able to inhibit the activity of MMLV reverse transcriptase. In particular, we suggest that this inhibition was due to a competitive interaction of the nucleopeptide at the primer site and was related to the ability of **3** to form complexes with polyA, similarly to other examples already described in literature (Moggio et al. 2007; Roviello et al. 2010). Finally, taken together, all these findings are in favour of the use of alternated 4-piperidyl glicine/L-arginine nucleopeptides, synthesizable in solid phase with an appropriate sequence and number of bases, as novel tools able to interact with RNA which could be beneficial in the medical research.

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