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ORIGINAL ARTICLE

Synthesis and aggregation properties of a novel enzymatically resistant nucleoamino acid

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Abstract In this work, we describe the synthesis, evaluation of some biological properties, such as DNA- and RNA-binding ability and *in sero* stability, as well as the supramolecular assembly of a novel nucleoamino acid based on L-spinacine. More particularly, a thymine-containing L-spinacine derivative was synthesized in liquid phase by a simple peptide-coupling procedure. Subsequently, nucleic acid and Cu^{2+}-binding ability, as well as self-assembly properties of the novel nucleoamino acid, were investigated by spectroscopy (CD and UV) and laser light scattering which furnished interesting information on the assembly of supramolecular networks based on the peptidyl nucleoside analog. Finally, nucleoamino acid enzymatic stability was studied and a half life of about 7 days was found in the presence of fresh human serum.

Keywords Spinacine · Nucleobase · Supramolecular · DNA

Introduction

In the last decades, considerable relevance has been attributed to hydrogels, as well as water-soluble macromolecular networks, useful for various technical and biomedical purposes (Shuai et al. 2005). These polymeric systems, based on one or more types of monomeric units, are formed by non-covalent bonding occurring between the molecular subunits. Many classes of molecules able to form supramolecular architectures are reported in several literature examples. For example, it is worth mentioning polynucleobase-molecules such as nucleic acids, particularly relevant in nanomedicine (Chhabra et al. 2010), peptide nucleic acids (Amato et al. 2009) and nucleopeptides (Roviello et al. 2011a), as well as monomeric units bringing a single nucleobase (Snip et al. 2002; Roviello et al. 2011b) which can form supramolecular systems based on the interaction between the nucleobases. Supramolecular assembly was also reported for a family of structures based on the non-covalent interaction between carboxylic acids and imidazole or imidazole-containing structures (carboxylic acid–imidazole synthon, Fig. 1) (Aakeröy and Salmon 2005).

Taking into account the importance of structural elements like DNA bases, carboxylic acid and imidazole groups in the non-covalent polymerization of molecules, we designed and realized a novel molecule the structure of which incorporated all of these three moieties, with the aim to obtain a novel supramolecular device. In other words, we realized a thymine–based nucleoamino acid (Fig. 2), characterized by a L-spinacine residue carrying the DNA nucleobase through its *N*-α-amino group (*N*-α).

Interestingly, the structure of the naturally occurring L-spinacine (Remelli et al. 1997) contains simultaneously both an imidazole ring and a carboxylic acid moiety which are useful for the supramolecular recognition based on the well-established carboxylic acid–imidazole synthon. Furthermore, the use of this histidine cyclic analogue, characterized by many biological properties and able to form complexes with Cu (II) cations, allows for the obtainment

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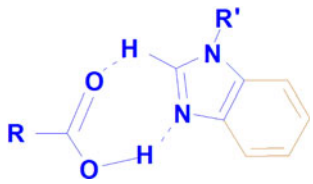

Fig. 1 Representation of hydrogen bonds in a carboxylic acid–imidazole synthon

of enzymatically stable compounds useful for biomedical applications (Blankley et al. 1991; Braibanti et al. 1973; Conato et al. 2001).

Materials and methods

Abbreviations

The following abbreviations are used: circular dichroism (CD), *N*,*N*-diisopropylethylamine (DIEA), dimethyl sulfoxide (DMSO), *O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HATU), nuclear magnetic resonance (NMR), thymin-1-yl acetic acid (TCH_2COOH), trifluoroacetic acid (TFA), thin layer chromatography (TLC), 2,4,6-trimethylpyridine (TMP), ultra violet (UV).

Chemicals

L-4,5,6,7-Tetrahydro-1H-imidazo[4,5-c] pyridine-6-carboxylic acid (L-spinacine) was purchased from Bachem. HATU was ABI; $CuCl_2{\cdot}2H_2O$ was Aldrich. Anhydrous DMSO, DIEA and solvents for HPLC chromatography were purchased from Romil. TFA, TMP, PolyA, and dA_{12} were Biomers. Chromatography glass plates for TLC were purchased from Machery-Nagel.

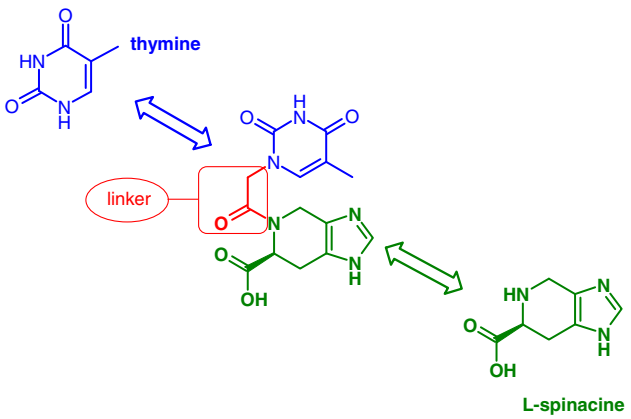

Fig. 2 Representation of the structure of the novel nucleoamino acid

Apparatus

The nucleoamino acid 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 (320°C), and with a complete Surveyor HPLC system, comprising an MS pump, an autosampler, and a PDA detector, using a Phenomenex Jupiter C18 300 Å (5 µm, 4.6 × 150 mm) column. Gradient elution was performed (monitoring at 260 nm) by building up a gradient starting with buffer A (0.05% TFA in water) and applying buffer B (0.05% TFA in acetonitrile) with a flow rate of 0.8 ml/min. High resolution mass spectra (HRMS) were acquired on a linear ion trap LTQ Orbitrap XL hybrid Fourier Transform MS (FTMS) equipped with an ESI ION MAX source (Thermo-Fisher, San Josè, CA, USA). Specific rotation was measured on a Perkin-Elmer 243 B polarimeter. ^{1}H NMR spectrum was recorded at 25°C on a 400 MHz Varian spectrometer. Chemical shifts (δ) are given in parts per million (ppm). Proton chemical shifts were referenced to residual CHD_2SOCD_3 ($\delta = 2.55$, quin) signals. HPLC 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 (5 mm, 4.6 × 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 1 ml/min. Samples containing the nucleoamino acid 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, whereas ultraviolet (UV) spectra were recorded on a UV–Vis Jasco model V-550 spectrophotometer, both equipped with a Peltier ETC-505T temperature controller, using a Hellma quartz cell with a light path of 10 mm, and a Hellma 238-QS tandem quartz cell (2 × 0.4375 cm). For light scattering a MiniDAWN Treos spectrometer (Wyatt Instrument Technology Corp.) equipped with a laser operating at 658 nm was used in batch mode (off-line).

Synthesis of the L-spinacine-based nucleoamino acid

The novel nucleo amino acid was synthesized in liquid phase starting from the commercial L-spinacine and TCH_2COOH. More in detail, L-spinacine (1 eq, 50 mg, 0.30 mmol) was solved in anhydrous DMSO (1 ml), treated with DIEA (0.6 eq, 31 µl, 0.18 mmol) and TMP (0.6 eq, 25 µl, 0.18 mmol) and subsequently reacted with TCH_2COOH (2 eq, 119 mg, 0.60 mmol) which was previously preactivated with HATU (1.9 eq, 217 mg, 0.57 mmol) and DIEA (2 eq, 102 µl, 0.60 mmol)/TMP (2 eq, 80 µl, 0.60 mmol) in DMSO (1 ml) for 2 min

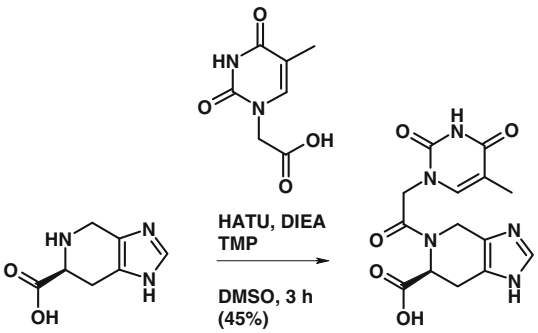

Fig. 3 Schematic representation of the synthetic strategy adopted for the obtainment of the L-spinacine nucleoamino acid

(Fig. 3). After 3 h, the reaction mixture was treated with 500 ml of water and after freezing the sample was lyophilized. The crude material was then treated with water and purified by RP-HPLC on a Phenomenex Juppiter C18 300 Å (5 mm, 4.6 × 250 mm) column using a linear gradient of 2% (for 5 min) to 80% B' in A' over 20 min (1.0 ml/min); $t_R = 6.79$ min. The precipitation of a white compound was observed in the aqueous solution containing the crude sample to be purified by HPLC. After HPLC and ESI MS analysis, it was possible to ascertain that this compound was identical to the reaction product already recovered by HPLC purification. Considering the amount of sample recovered by both HPLC and precipitation the desired product was obtained with an overall yield of 45%. $[\alpha]_D = -120.1°$ (c 0.04, d_6-DMSO); LC-ESI MS method: 2% (5 min) to 30% B in A over 10 min; $t_R = 6.14$ min; *m/z*: 333.27 (found), 334.31 (expected for $[C_{14}H_{15}N_5O_5 + H]^+$); High-resolution Mass Spectrum (ESI^+). *m/z*: 334.1144 (found), 334.1151 (expected for $[C_{14}H_{15}N_5O_5 + H]^+$); 356.0964 (found), 356.0971 (expected for $[C_{14}H_{15}N_5O_5 + Na]^+$); TLC Rf 0.27 ($CH_2Cl_2$:MeOH:DIEA = 6.9:3.0:0.1) NMR δ_H (400 MHz, DMSO-d_6) 13.35 (1H, bs, COOH), 11.39 (1H, s, NH thymine), 8.40 and 8.19 (1 H, s, Z- and E-amide conformers, aromatic $CH_{spin}NH$), 7.41 (1H, s, aromatic CH thymine), 5.63-4.40 (5H, m, NCH_2CO, NCH_2C_{spin}, NH_{spin}), 4.08-2.94 (3H, m, CH_{alpha}, CH_2CH_{alpha}), 1.80 (3H, s, CH_3 thymine).

Static light scattering

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

Computational methods

The molecular volume (V) of a single nucleoamino acid unit was estimated to be 208.8 $Å^3$ (radius = $[3V/4\pi]^{1/3}$ = 3.68 Å) by WebLab ViewerPro 3.7 software (Molecular Simulations, Inc., San Diego-CA, 2000) in analogy to other recent literature examples (Lindquist et al. 2010; Cangelosi et al. 2010).

UV and CD studies

Copper (II) binding studies were performed in a Hellma 115-QS quartz cell with a light path of 10 mm (400 μl) on a 50 μM nucleoamino acid solution by using a 83 mM $CuCl_2 \cdot 2H_2O$ solution. All the CD spectra were recorded using the following parameters: scanning speed, 50 nm/min; 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 Hellma 238-QS tandem quartz cell (2 × 0.4375 cm) tandem cell.

Human plasma stability assay

7 μl of nucleoamino acid solution (2.3 mM) was added to 93 μl of 100% fresh human serum in a micro-vial and the mixture was incubated at 37°C. Aliquots (10 μl) were taken at 0, 1, 2, 3, 4, 5, 24 and 168 h, quenched by adding 10 μl of a 7 M urea solution, kept at 95°C for 2 min and then stored at −20°C until subsequent analysis. The withdrawn samples were analyzed by HPLC on a Phenomenex Juppiter C18 300 Å (5 mm, 4.6 × 250 mm) column using a linear gradient of 2% (for 5 min) to 80% B in A over 20 min (1.0 ml/min).

Results and discussion

We realized a convenient and fast synthetic route to the chiral nucleo-L-spinacine monomer, in which the L-1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid moiety is connected to the DNA nucleobase by an amide bond, suitable also for the *N*-terminus derivatization of peptides and nucleopeptides by solid phase synthesis. The nucleobase-containing monomer was synthesized starting from the commercially available L-spinacine and thyminyl acetic acid in DMSO by using HATU as coupling reagent and DIEA/TMP as bases (Fig. 3).

After HPLC purification, the identity of the desired product was established by LC-ESIMS (Fig. S1, ESI†), HRMS and NMR. Interestingly, the route to the nucleoamino acid makes use of the unprotected amino acid and results fast and convenient. Moreover, it could be also suitable for the synthesis of all four nucleobase-containing L-spinacine derivatives.

Static laser light scattering

Molecular self-assembly of the nucleoamino acid was investigated by static laser light scattering. The experiments were performed in triplicate on a 50 μM nucleoamino acid solution in 10 mM phosphate buffer at pH 7.5. The data collected were in accordance with the formation of a supramolecular network with a mean hydrodynamic radius of about 32 nm (320 Å). Considering a radius of 3.68 Å for a single nucleoamino acid molecule, as computed by WebLab ViewerPro 3.7 software in analogy to other recent literature examples (Lindquist et al. 2010; Cangelosi et al. 2010), it can be deduced that more than eighty molecules are present in the interior of the supramolecular networks revealed in the solution by laser scattering. Moreover, on the basis of the molecular recognition already demonstrated for the carboxylic acid–imidazole synthon, we propose the multiple interaction scheme involving the L-spinacine-based nucleoderivatives represented in Fig. 4, in which both nucleobase–nucleobase and COOH–imidazole interactions, reinforced also by further hydrogen bonds, play a key role.

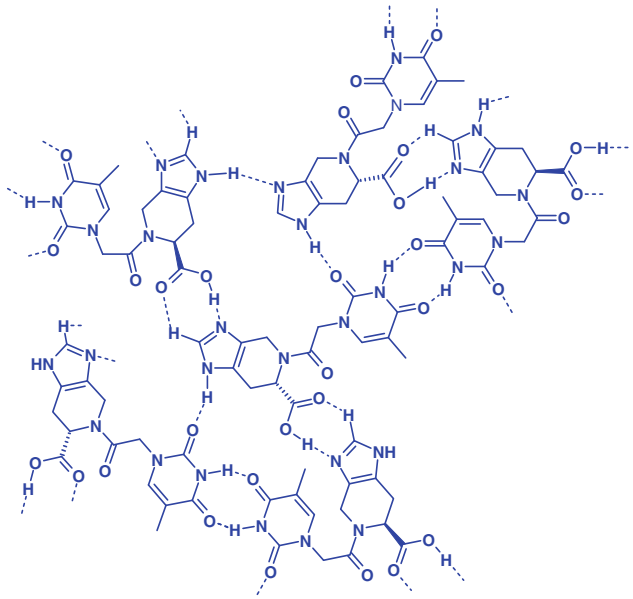

Fig. 4 Schematic representation of the multiple interaction scheme involving the L-spinacine-based nucleoamino acid units proposed by us

Metal ion binding studies

Nearly all plants, animals and bacteria use copper; moreover, copper is the third most abundant transition element in the human body after zinc and iron. As reported in literature, interbase Cu^{2+} coordination can replace the hydrogen bonding schemes found in the natural base pairs dA:dT and dG:dC (Meggers et al. 2000). This type of interaction is at the basis of supramolecular structures, of possible utility for the realization of novel biomaterials, governed by the cation–nucleobase recognition found for example in complexes of Cu^{2+} with nucleoamino acids, nucleopeptides, nucleosides and nucleotides (Mizutani et al. 1999; Kuesel et al. 2005; Armentano et al. 2007; Santangelo et al. 2007). Interestingly, copper (II)-peptide complexes with biological activity were evidenced for the natural tripeptide glycyl-L-histidyl-L-lysine (GHK) and its L-spinacine-based analogs which act as cell growth factors (Conato et al. 2001). In order to investigate the influence of copper (II) ions on the supramolecular networks revealed by laser light scattering for the systems based on the thymine-containing L-spinacine derivative, we performed UV experiments in which the absorbance of a 50 μM solution of nucleoamino acid, in the presence of phosphate buffer at pH 7.5, was recorded after the addition of increasing amounts of a $CuCl_2{\cdot}2H_2O$ solution.

As can be seen in Fig. 5, the UV absorbance due to the thymine moiety decreases (6a and b) in the presence of Cu (II) cations, suggesting an interaction of the metal ion with the nucleobase which causes a clear stacking effect. In other words the L-spinacine nucleoamino acid, which is intrinsically able to form supramolecular networks, shows also a base stacking effect in the presence of Cu^{2+}, an interesting feature which may open up new possibilities for the obtainment of nucleoamino acid-metal hybrid materials governed by non-covalent nucleoamino acid–metal ion interactions.

CD studies

In order to characterize by CD the novel nucleoamino acid, we recorded in the 190–320 nm wavelengths range the CD spectrum relative to a 10 μM solution of nucleoamino acid (in 10 mM phosphate buffer, pH 7.5, 5°C) and observed a positive band centered at 245 nm probably associated to the aromatic moiety of the molecule (Fig. S2, ESI†). Regarding the binding with DNA, CD experiments were conducted at 5°C in a tandem cell (1 + 1 ml) (Roviello et al. 2011c) recording in the 210–320 nm wavelengths range the ''sum'' spectrum of the separated components and the ''mix'' spectrum, recorded after mixing the two ligand solutions (in 10 mM phosphate buffer, pH 7.5), relative to 1 equivalent in nucleobase of dA_{12} with respect to thyminyl nucleoamino acid (100 nmol). No appreciable

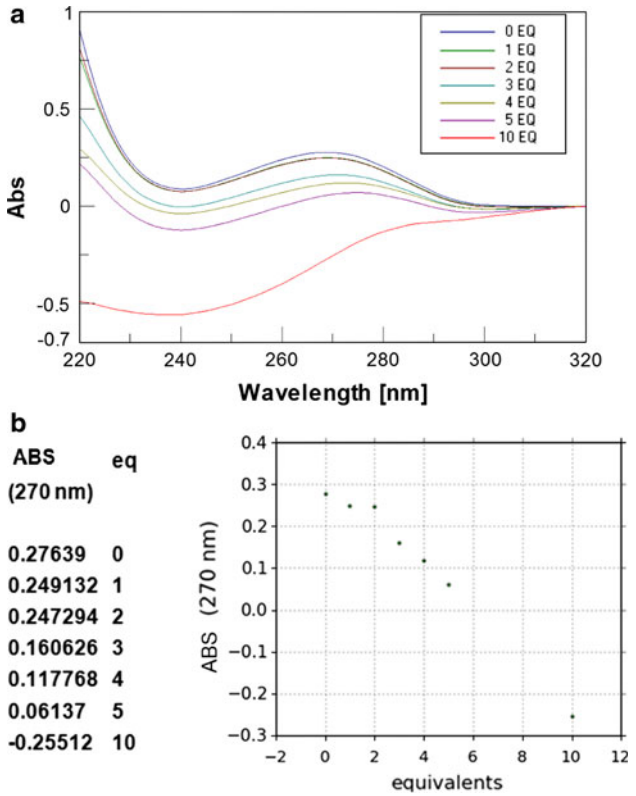

Fig. 5 **a** UV spectra relative to a 50 μM solution of thyminyl nucleoamino acid (10 mM phosphate buffer, pH 7.5, 25°C) in the presence of 0, 1, 2, 3, 4, 5 and 10 equivalents of Cu (II), **b** plot of UV absorbance values at 270 nm as a function of the amount (equivalents) of Cu^{2+}

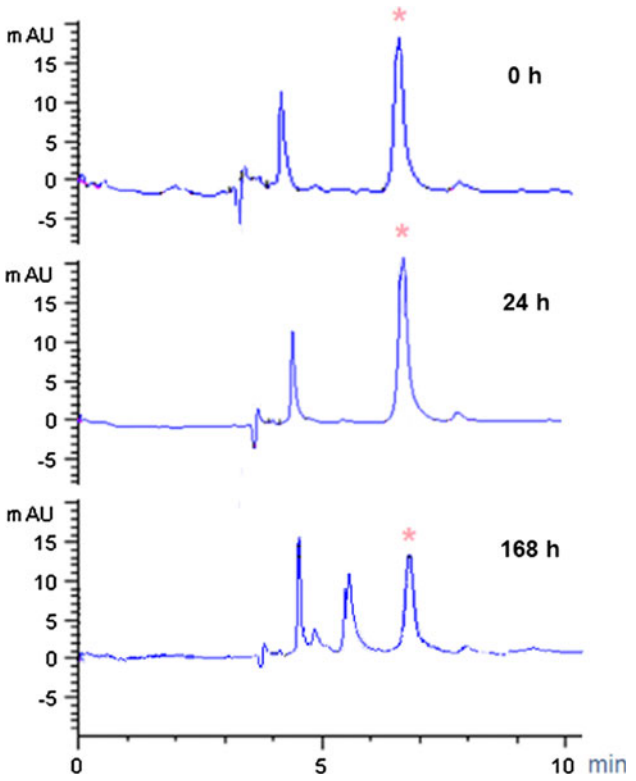

Fig. 6 RP-HPLC analysis of the serum stability assay on the nucleoamino acid

difference was observed between the ''sum'' (green line) and ''mix'' (blue) CD spectra indicating that no significant interaction took place between the nucleoamino acid and DNA (Fig. S3, ESI†). On the other hand, no interaction with RNA (polyA) was revealed by an analogous experiment performed in the same experimental conditions (Fig. S4, ESI†).

Human serum stability assay

The enzymatic resistance of this novel class of nucleoamino acids was investigated by incubating the nucleoamino acid in fresh human serum at 37°C and analyzing by RP-HPLC samples withdrawn from the reaction mixture at various times. By analyzing the results of the stability assay (Fig. 6), it can be deduced that the nucleoamino acid is highly stable and presents an half life of about 168 h.

Conclusion

In conclusion, in this work we realized the synthesis, purification and characterization of a novel nucleoamino acid based on a L-spinacine residue bearing the DNA nucleobase anchored to its *N*-α. Preliminary UV and LS studies on various nucleoamino acid solutions, as well as a CD investigation of the interaction of the novel nucleoamino acid with DNA and RNA, were performed in order to evaluate the formation of supramolecular networks, based on nucleobase recognition. From UV and LS studies it was possible to demonstrate the formation of molecular networks, in which nucleoamino acid units were held together by weak interactions (probably H-bonding, hydrophobic and aromatic interactions), whose structure changed as a result of the interaction with metal ions (Cu^{2+}). Furthermore, the enzymatic stability of the novel peptidyl nucleoside analogue was also demonstrated by experiments with fresh human serum. All these findings strongly encourage further investigations on the novel class of nucleoamino acids presented in this work, in view of their possible employment as building blocks of supramolecular assemblies for biomedical applications.

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

References

Aakeröy CB, Salmon DJ (2005) Building cocrystals with molecular sense and supramolecular sensibility. Cryst Eng Commun 7:439–448

Amato J, Oliviero G, De Pauw E, Gabelica V (2009) Hybridization of short complementary PNAs to G quadruplex forming oligonucleotides: an electrospray mass spectrometry study. Biopolymers 91:25–244

Armentano D, Mastropietro TF, Julve M, Rossi R, Rossi P, De Munno G (2007) A new octanuclear copper(II)-nucleoside wheel. J Am Chem Soc 129:2740–2741

Blankley CJ, Hodges JC, Klutchko SR, Himmelsbach RJ, Chucholowski A, Connolly CJ, Neergaard SJ, Van Nieuwenhze MS, Sebastian A, Quin J III, Essenburg AD, Cohen DM (1991) Synthesis and structure–activity relationships of a novel series of non-peptide angiotensin II receptor binding inhibitors specific for the AT2 sub-type. J Med Chem 34:3248–3260

Braibanti A, Dallavalle F, Leporati E, Mori G (1973) Acid–base properties of spinaceamine and spinacine and their complexing capacity with divalent metals. J Chem Soc Dalton Trans 1973:323–328

Cangelosi VM, Zakharov LN, Johnson DW (2010) Supramolecular "transmetalation" leads to an unusual self-assembled P2L3 cryptand. Angew Chem Int Ed 49:1248–1251

Chhabra R, Sharma J, Liu Y, Rinker S, Yan H (2010) DNA self-assembly for nanomedicine. Adv Drug Deliv Rev 62:617–625

Conato C, Gavioli R, Guerrini R, Kozlowski H, Mlynarz P, Pasti C, Pulidori F, Remelli M (2001) Copper complexes of glycyl-histidyl-lysine and two of its synthetic analogues: chemical behaviour and biological activity. Biochim Biophys Acta 1526(2):199–210

Kuesel A, Zhang J, Alvarino Gil M, Stueckl CA, Meyer-Klaucke W, Meyer F, Diederichsen U (2005) Metal binding within a peptide based nucleobase stack with tuneable double strand topology. Eur J Inorg Chem 431:7–4324

Lindquist NR, Carter TG, Cangelosi VM, Zakharov LN, Johnson DW (2010) Three's company: co-crystallization of a self-assembled S(4) metallacyclophane with two diastereomeric metallacycle intermediates. Chem Commun 46:3505–3507

Meggers E, Holland PL, Tolman WB, Romesberg FE, Schultz PG (2000) A novel copper-mediated DNA base pair. J Am Chem Soc 122:10714–10715

Mizutani M, Kubo I, Jitsukawa K, Masuda H, Einaga H (1999) Nucleobase stacking evidenced on ternary metal (palladium(II), copper(II)) complexes with nucleobasi amino acids and aromatic diimines. Inorg Chem 38:420–421

Remelli M, Pulidori F, Guerrini R, Bertolasi V (1997) Synthesis of spinacine and spinacine derivatives: crystal and molecular structures of *N*π-hydroxymethyl spinacine and *N*α-methyl spinaceamine. J Chem Crystallogr 27:507–513

Roviello GN, Musumeci D, Bucci EM, Pedone C (2011a) Evidences for supramolecular organization of nucleopeptides: synthesis, spectroscopic and biological studies of a novel dithymine L-serine tetrapeptide. Mol BioSyst 7:1073–1080

Roviello GN, Ricci A, Bucci EM, Pedone C (2011b) Synthesis, biological evaluation and supramolecular assembly of novel analogues of peptidyl nucleosides. Mol BioSyst. doi: 10.1039/c1mb05007a

Roviello GN, Di Gaetano S, Capasso D, Franco S, Crescenzo C, Bucci EM, Pedone C (2011c) RNA-binding and viral reverse transcriptase inhibitory activity of a novel cationic diamino acid-based peptide. J Med Chem 54:2095–2101

Santangelo MG, Medina-Molner A, Schweiger A, Mitrikas G, Spingler B (2007) Structural analysis of Cu(II) ligation to the 5'-GMP nucleotide by pulse EPR spectroscopy. J Biol Inorg Chem 12(6):767–775

Shuai X, Merdan T, Unger F, Kissel T (2005) Supramolecular gene delivery vectors showing enhanced transgene expression and good biocompatibility. Bioconjug Chem 16:322–329

Snip E, Koumoto K, Shinkai S (2002) Gel formation properties of a uracil-appended cholesterol gelator and cooperative effects of the complementary nucleobases. Tetrahedron 58:8863–8873