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A novel synthetic luteinizing hormone-releasing hormone (LHRH) analogue coupled with modified β-cyclodextrin: Insight into its intramolecular interactions

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ABSTRACT

Background: Cyclodextrins (CDs) in combination with therapeutic proteins and other bioactive compounds have been proposed as candidates that show enhanced chemical and enzymatic stability, better absorption, slower plasma clearance and improved dose–response curves or immunogenicity. As a result, an important number of therapeutic complexes between cyclodextrins and bioactive compounds capable to control several diseases have been developed.
Results: In this article, the synthesis and the structural study of a conjugate between a luteinizing hormone-releasing hormone (LHRH) analogue, related to the treatment of hormone dependent cancer and fertility, and modified β-cyclodextrin residue are presented. The results show that both the phenyl group of tyrosine (Tyr) as well as the indole group of tryptophan (Trp) can be encapsulated inside the cyclodextrin cavity. Solution NMR experiments provide evidence that these interactions take place intramolecularly and not intermolecularly.
Conclusions: The study of a LHRH analogue conjugated with modified β-cyclodextrin via high field NMR and MD experiments revealed the existence of intramolecular interactions that could lead to an improved drug delivery.
General significance: NMR in combination with MD simulation is of great value for a successful rational design of peptide–cyclodextrin conjugates showing stability against enzymatic proteolysis and a better pharmacological profile.

1. Introduction

Peptides and proteins are well known weaponry against serious diseases. Thanks to advances in biotechnology, the production of these molecules on a large-scale has been accelerated. However, this huge impetus given in: a) the synthesis and development of novel synthetic peptides and b) the progress in molecular biology, has not found a parallel in the development of delivery systems for this class of molecules [1]. It is well known that peptides suffer from chemical and enzymatic instability, poor absorption through biological membranes, rapid plasma clearance, peculiar dose response curves and immunogenicity [2].

A great effort is made by the scientific community in order to eliminate these drawbacks of peptides and proteins. An interesting approach is to administer peptides and proteins in combination with cyclodextrins (CDs). Actually, this approach is not limited only to the class of peptide molecules but also to other smaller size bioactive molecules which suffer from similar problems [3–5]. Partial encapsulation of a

Abbreviations: BOP, benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate; CLTR-Cl, 2-chlorotrityl chloride; DCC, N,N′-dicyclohexylcarbodiimide; DMA, dimethylacetamide; DIC, N,N′-diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMF, N,N-dimethylformamide; ESI-MS, electrospray ionization mass spectrometry; Fmoc, 9-fluorenylmethyloxycarbonyl; HBTU, N,N,N′,N′-tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; MD, molecular dynamics; NHS, N-hydroxysuccinimide; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; RP-HPLC, reversed phase high performance liquid chromatography; TBTU, O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; TOCSY, total correlation spectroscopy

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bioactive molecule inside the CD cavity is the basis of the approach. Thus, it is reported in the literature that CDs can protect peptides and proteins against enzymatic as well as chemical degradation [6].

CDs are a family of cyclic oligosaccharides, and the most common which are commercially available consisting of six (α-CD), seven (β-CD) or eight (γ-CD) α-1,4-linked glucosidic bonds. Their shape is toroidal or cone-shaped, due to the absence of free rotation of the bonds linking the glucose units. Each glucose unit has three hydroxyl groups, two secondary connected at carbons 2 and 3 of the glucose unit and one primary connected at carbon 6. Thus, a total number of twenty one hydroxyl groups are present in β-CD and their presence is mainly responsible for CD water solubility. The primary hydroxyl groups are located in the narrow rim of the cone, while secondary hydroxyl groups are located in the wide rim. On the other hand, the interior of CDs is relatively hydrophobic due to the presence of ether oxygens at the C4 and hydrogens attached at carbons C3 and C5. This allows CDs to entrap hydrophobic molecules [7]. α-CD may typically complex low molecular weight molecules or compounds with aliphatic side chains, β-CD can complex aromatic and heterocyclic molecules, and γ-CD can form inclusion complexes with molecules as large as macrocycles, steroids and C_{60} [2,8].

In peptides, encapsulation of the whole molecule inside any type of CD is not possible, since peptides are usually much larger compared to CDs. However, as with other types of drugs, inclusion of a small part of the peptide can take place and this process in certain cases can reduce the above mentioned problems like enzymatic and chemical degradation [2]. A carefully designed attachment of the CD unit could leave parts of the peptide, important to the binding process, free of encapsulation and as a result would permit the drug to retain its bioactivity. Also, the use of modified CDs can greatly alter drug lipophilicity and enhance transport through membranes.

CDs and CDs based systems have tested for the carriage of many anticancer drugs [9]. The gonadotropin-releasing hormone (GnRH) known as luteinizing-hormone-releasing hormone (LHRH) stimulates the secretion of gonadotropins via binding to receptors on the surface of gonadotrophin cells of the anterior pituitary. It is a decapeptide, amide (pGlu–His–Trp–Ser–Tyr–Gly–Leu–Arg–Pro–Gly–NH_2) produced in the hypothalamus and adjusts the steroidogenesis and gametogenesis. The gonadotropins (LH, luteinizing hormone and FSH, follicle-stimulating hormone) regulate the production of gametes and steroid sex hormones from the gonads (ovaries and testes) [10,11].

Over the last years the synthesis of various LHRH peptide analogues (agonists or antagonists) introduced new perspectives in the treatment of hormone dependent cancer and fertility. Leuprolide (pGlu–His–Trp–Ser–Tyr–DLeu–Leu–Arg–Pro–NHEt) is a synthetic altered peptide ligand (APL) of LHRH. Leuprolide has an amino acid skeleton and thus is sensitive to proteolysis. Sensitivity is enhanced further by the fact that Leuprolide has low intestinal absorption and bioavailability (less than 1%). In general, three mainly peptide bonds of the LHRH analogues are more sensitive to hydrolysis, namely Trp^3–Ser^4, Ser^4–Tyr^5 and Tyr^5–Gly^6 [12,13].

Aiming the reduction of the proteolysis sensitivity, we report here, for the first time, the synthesis and conformational study of a LHRH analogue coupled with 3-monoamino-β-CD. The synthesized LHRH analogue is based on the Leuprolide sequence (DLeu instead of Gly at position 6) and the intramolecular interactions between the peptide and the CD moiety were explored via a high field 2D NMR.

2. Materials and methods

2.1. Materials

2-Chlorotrityl chloride polystyrene resin (1% DVB, 200–400 mesh), Fmoc–εAhx–OH, Fmoc–Arg(Pbf)–OH, Fmoc–Gly–OH, Fmoc–Leu–OH, Fmoc–DLeu–OH, Fmoc–Ser(tBu)–OH, Fmoc–Tyr–OH, Fmoc–Trp–OH, Fmoc–His(Trt)–OH, pGlu–OH and Fmoc–Pro–OH were purchased from Chemical and Biopharmaceutical Laboratories of Patras, Patras, Greece. 3-Monoamino-β-CD hydrate was purchased from TCI Europe. All solvents and other reagents were purchased from Merck, Sigma-Aldrich and Fluka companies. DC-Alufolien Kieselgel 60 F254 (Merck) was used for Thin-layer chromatography (TLC) analysis of synthetic products. Water which used in all experiments was purified with Milli-Q Water Purification System and the resistivity of the treated water is more than 18 MegaΩ/cm. Peptide **2** and the peptide conjugate **3** were purified by semi-preparative reverse phase high performance liquid chromatography (RP-HPLC) with a Waters system equipped with a 600E controller and a Waters 996 photodiode array UV detector. The analysis was controlled by an operating Millenium 2.1 system and a Lichrosorb C18 reversed phase column (250 × 10 mm with 7 nm packing material). Electron spray ionization mass spectroscopy (ESI-MS) experiments were performed with a TSQ 7000 spectrometer (Electrospray Platform LC of Micromass) coupled to a MassLynx NT 2.3 data system.

2.2. Synthesis

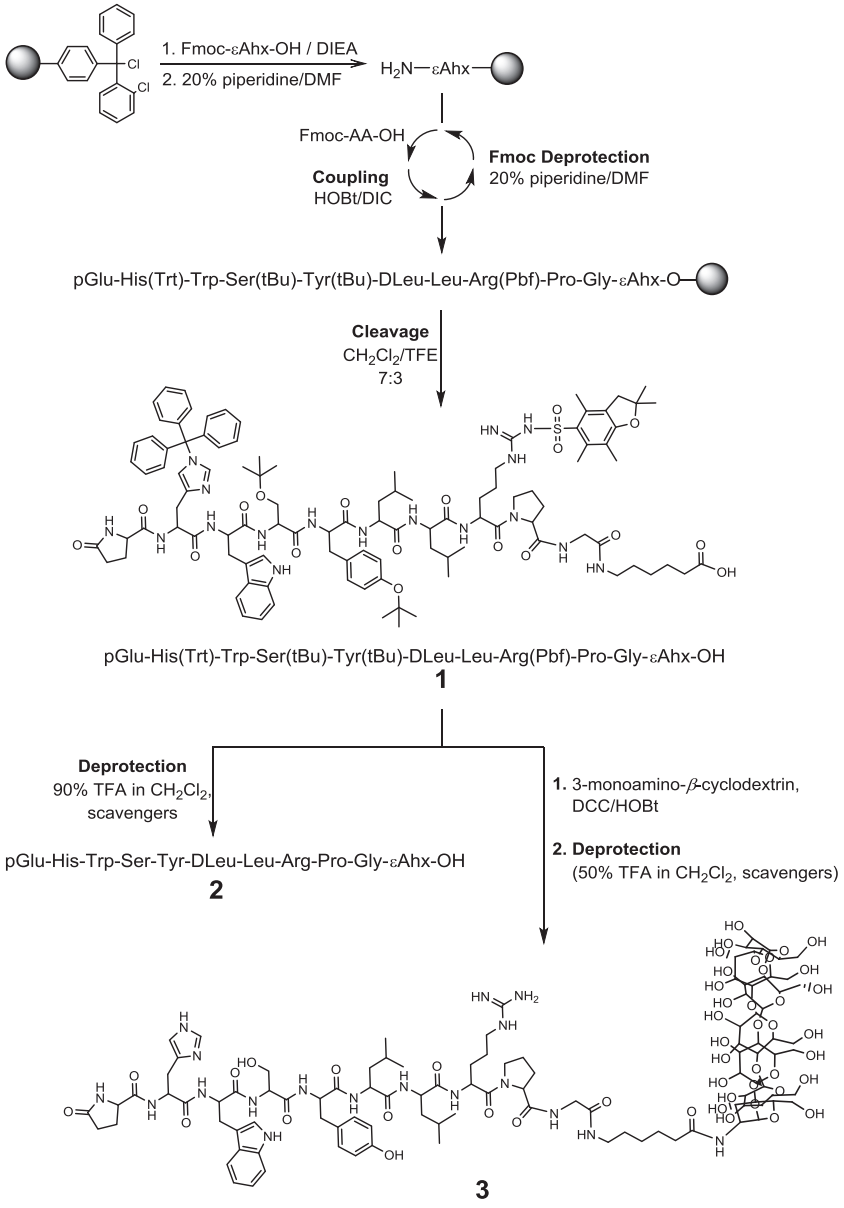
The synthetic approach for the synthesis of the LHRH analogue **2** and the target molecule **3** is depicted in Scheme 1.

2.2.1. Synthesis of protected peptide *1*

The peptide was synthesized following the Fmoc/tBu solid phase methodology utilizing CLTR-Cl resin (1.500 g, 2.25 mmol). The first N^{α}-Fmoc-protected amino acid, Fmoc–εAhx–OH (1 equiv.), was coupled (esterified) to the resin in the presence of DIEA (4.5 equiv.) in CH_2Cl_2 (15 mL) for 1 h at room temperature (RT). Subsequently were added CH_3OH (1.5 mL) and DIEA (0.75 mL) and the mixture was stirred for another 30 min at RT. The Fmoc–εAhx–resin was successively filtered and washed with a mixture of CH_2Cl_2/CH_3OH/DIEA (80:15:5, 3 × 15 mL 5 min), DMF (5 × 15 mL) and *i*PrOH (3 × 15 mL). The remaining protected peptide chain was coupled with the appropriate amino acids (2.5 equiv.) in the presence of DIC (2.75 equiv.) and HOBt (3.75 equiv.) in DMA for 4–6 h followed by Fmoc deprotection with 3 × piperidine (20% in DMF) for 5, 15 and 10 min, respectively. The following Fmoc protected amino acids were used for the synthesis: Fmoc–Gly–OH, Fmoc–Pro–OH, Fmoc–Arg(Pbf)–OH, Fmoc–Leu–OH, Fmoc–DLeu–OH, Fmoc–Tyr(tBu)–OH, Fmoc–Ser(tBu)–OH, Fmoc–Trp–OH, Fmoc–His(Trt)–OH and pGlu–OH. Completeness of each coupling and deprotection cycle was verified by the Kaiser test and TLC (1-BuOH/AcOH/H_2O 4:1:1 and CH_3CN/H_2O 5:1). The synthesized protected peptide on the resin was dried under vacuum and then was cleaved with CH_2Cl_2/TFE (7:3) for 2 h at RT (solution pH: 5–6). The mixture was filtered, the solvent was removed on a rotary evaporator and the obtained oily product was precipitated by the addition of cold diethyl ether (Et_2O) as an amorphous white solid (**1**) (0.285 g).

2.2.2. Synthesis and purification of deprotected peptide *2*

The deprotection of protected peptide **1** (0.035 g) was achieved using 90% TFA in CH_2Cl_2 solution in the presence of 0.3% triethylsilane, anisole and H_2O as scavengers for 4 h at RT. The solvent was partially evaporated and the crude product was precipitated with cold Et_2O and collected by filtration (0.0272 g). The crude product was further purified by semi-preparative RP-HPLC (column: Lichrosorb C 18, 7 μm, 250 × 10 mm, eluents: A: 0.08% TFA/H_2O, B: 0.08% TFA/CH_3CN, gradual gradient: from 10% to 60% B in 45 min, flow rate: 3 mL/min, detection: 230 nm, 254 nm, identification with ESI-MS). The purity of the final product **2** was assessed by analytical RP-HPLC (column: Lichrosorb C18, 5 μm, 250 × 4.6 mm, eluents: A: 0.08% TFA/H_2O, B: 0.08% TFA/CH_3CN, gradual gradient: from 10% to 100% B in 30 min, flow rate: 1 mL/min, detection: 214 nm or 254 nm) and the purity was higher than 98% (0.011 g, 46%). The final product **2** was identified by ESI-MS m/z: calcd for $C_{65}H_{94}N_{17}O_{15}$ $[M + H]^+$ 1352.70, found 1352.71.



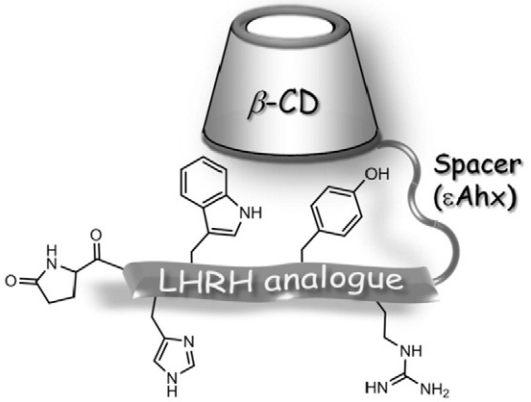
Scheme 1. Total synthesis of the LHRH analogue (**2**) and its conjugate with 3-monoamino-β-CD (**3**).

*2.2.3. Synthesis and purification of the peptide–β-CD conjugate **3***

Side chain-protected peptide pGlu–His(Trt)–Trp–Ser(tBu)–Tyr(tBu)–DLeu–Leu–Arg(Pbf)–Pro–Gly–εAhx–OH (**1**) (0.035 g, 1.79×10^{-2} mmol) and HOBt (0.006 g, 2.5 equiv.) were completely dissolved with 0.5 mL DMF and after cooling at 0 °C DCC (0.009 g, 2.5 equiv.) was added. The solution was left for 30 min at 0 °C and then for 45 min at RT. Then, 3-monoamino-β-CD (0.022 g, 1.1 equiv.) was added and the clear colorless solution was left overnight at 45 °C. According to TLC (*i*PrOH/H_2O 7:3) at the end of the reaction almost all of the 3-monoamino-β-CD was reacted. Cold Et_2O (4 mL) was added to this solution producing immediate precipitation of an amorphous white solid which subsequently was filtered and washed with cold Et_2O (3 × 1 mL) (0.033 g, 60%). Deprotection of the protected peptide–β-CD conjugate (0.033 g, 1.07×10^{-2} mmol) was achieved using 50% TFA in CH_2Cl_2 solution under anhydrous conditions in the presence of 0.3% triethylsilane and anisole as scavengers for 4 h at RT. The solvent was partially evaporated and the crude product was precipitated with cold Et_2O and collected by filtration. The crude product was further purified by semi-preparative RP-HPLC (column Lichrosorb C18, 7 μm, 250 × 10 mm), eluents: A: 0.08% TFA/H_2O, B: 0.08% TFA/CH_3CN, gradual gradient: from 20% to 60% B in 45 min, flow rate: 3 mL/min, detection: 230 nm, 254 nm, identification with ESI-MS. The purity of the final product **3** was assessed by analytical RP-HPLC (column: Lichrosorb C18, 5 μm, 250 × 4.6 mm, eluents: A, 0.08% TFA/H_2O, B, 0.08% TFA/CH_3CN, gradual gradient: from 20% to 100% B in 30 min, flow rate: 1 mL/min, detection: 214 nm or 254 nm) and the purity was ≥98% (0.011 g, 42%). The final product **3** was identified by ESI-MS m/z: calcd for $C_{107}H_{163}N_{18}O_{48}$ $[M + H]^+$ 2468.08, found 2467.71.

2.3. NMR spectroscopy

DMSO-d_6 (99%+) was purchased from Alfa Aesar, Karlsruhe Germany, packed in ampoules of 1 mL. Water was purified with Milli-Q Water Purification System. Ultra precision 5 mm NMR tubes from Norell were used containing 2 mM peptide **2** or conjugated peptide **3** in 0.7 mL of a mixture H_2O/DMSO-d_6 (9:1). At this concentration no line broadening effects, which would indicate aggregation of peptides, were observed.



Scheme 2. Schematic representation of the components, present in the conjugated LHRH analogue.

For other concentrations used, the volume was kept constant at 0.7 mL. The high-resolution NMR spectra were recorded on a Varian DirectDrive 800 MHz spectrometer at 298 K. All data were collected in phase sensitive mode using pulse sequences and phase-cycling routines provided in Varian libraries of pulse programs. The DQF-COSY and $^{1}H–^{13}C$ HSQC were performed with gradients. The 2D homonuclear proton spectra were acquired with a spectral width of 8012 Hz, 2048–4096 data points in t_2, 4–32 scans, 512 complex points in t_1, and a relaxation delay of 1.5 s. The mixing times in NOESY were 75 ms and 150 ms and in TOCSY 60 ms. The $^{1}H–^{13}C$ HSQC spectra were recorded with ^{1}H spectral width of 8013 Hz, ^{13}C spectral width of 30166 Hz, 1024 data points in t_2, 32 scans, 128 complex points in t_1 and a relaxation delay of 1.5 s. Spectra were processed and analyzed with the FELIX 2007 software package from Felix NMR Inc.

2.4. Molecular modeling

2.4.1. Structure preparation

The structure of CD was obtained from the Cambridge Structural Database (CSD reference code: BUVSEQ02) [14]. The peptide sequence was built using Pymol. The complete structure of the conjugate **3** was prepared using Maestro 9.3.5 *(Schr dinger Suite 2012.2) [15]. The initial structure was subjected to energy minimization using MacroModel 9.9, the OPLS2005 force field and the Generalized Born/Surface Area (GB/SA) continuum solvation model (implicit water solvent). The Polak Ribiere Conjugated Gradient (PRCG) method was used for the minimization for a number of 500 maximum iterations and a convergence threshold of 0.001 kJ/(mol × Å) [16].

2.4.2. Molecular dynamics in implicit solvent

MD simulations were performed using the Stochastic Dynamics method of the MacroModel module [16]. The SHAKE procedure was used to constrain the bond lengths to their initial values. The temperature was set at 298.1 K to simulate the experimental conditions. The GB/SA solvation model was used at a constant dielectric for water. The MD simulations included a stage of 1 ns equilibration and 20 ns simulation. Unconstrained and constrained MD simulations were performed. The NMR derived restraints used, involved tyrosine to β-CD and tryptophan to β-CD with a force constant of 5 kJ/(mol × Å^2) and a (±10%) uncertainty.

2.4.3. Molecular dynamics in explicit solvent

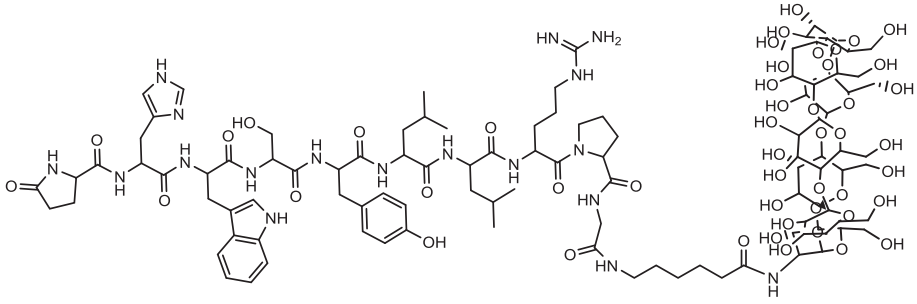
The MD calculations were performed using Desmond 3.4 [17]. The original system was solvated in a truncated octahedron box of size 14 Å in each direction by adding 2618 SPC water molecules. The system was brought in a local energy minimum using the steepest descent (until a gradient threshold of 25 kcal/(mol × Å) was obtained) and the limited-memory Broyden–Fletcher–Goldfarb–Shanno (LBFGS) algorithms [18]. The target temperature of 298 K was reached using simulated annealing simulation. The simulation time was set at 2 ns and the ensemble class to NVT. The MD simulation was performed in the NTP ensemble at 298.1 K and 1.01325 bar for 40 ns. A Langevin thermostat and barostat were used. The RESPA integrator was used with time step 2 fs for bonded and short-range interactions. The short range cut-off radius for the Coulombic interactions was set at 9 Å. The long range Coulombic interactions were calculated using the Smooth particle Mesh Ewald (PME) method.

3. Results and discussion

3.1. Molecular design

The synthesized LHRH analogue has DLeu at position 6, pyroglutamic amino acid as amino-terminal and glycine amino acid as carboxy terminal. The carboxyl group of glycine is an attractive candidate for the attachment of the CD unit. Unmodified CD, as a polyhydric alcohol, can react with carboxylic acid groups to produce esters as products. However, this reaction is complicated by the fact that β-CD has 21 hydroxyl groups. Thus, a commercially available, modified CD was used instead, where one of the secondary hydroxyl groups has been substituted by an amino group (3-monoamino-β-CD). In this case, a linkage can be obtained through an easier to control reaction between a reactive ester and an amino group. For example, there are certain activated carboxylic derivatives which show enhanced reactivity and selectivity towards the amino than the hydroxyl group. In addition, in literature this synthetic methodology has been successfully applied in other examples where peptides were attached to CDs [19–28]. Another point of consideration was the short distance between the CD and the peptide if the amino CD derivative is directly attached to glycine. To overcome this difficulty a non-natural and flexible amino acid [ε-aminohexanoic acid, (εAhx)] was added to glycine (Schemes 2 and 3) [20,28,29].

The rationale behind this design was that the εAhx due to its flexible nature can induce intramolecular interactions between the β-CD and LHRH analogue segments. In addition, the εAhx would act as a spacer



Scheme 3. Structure of the synthesized LHRH analogue coupled with 3-monoamino-β-CD.

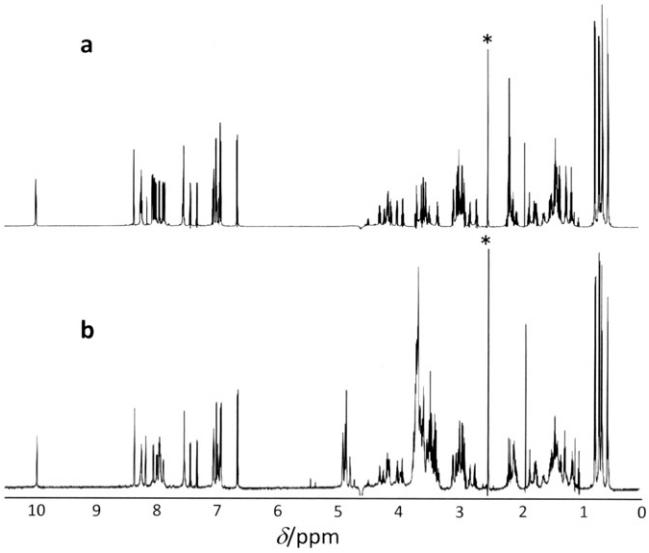


Fig. 1. ^{1}H NMR spectra (800 MHz, water/DMSO-d_6 (9:1), 298 K) of a: peptide **2**, b: conjugated peptide with β-CD **3**. "*" represents solvent peak.

reducing any aggregation and steric hindrance effects induced by the direct grafting of the peptide onto the CD.

In all previously described conjugates of other bioactive peptides with cyclodextrins [19–28], attachment of the CD unit takes place through the narrow rim of CD. In contrast, molecule **3** described in this work represents an example where a peptide skeleton is attached to CD via the large rim of CD. Substitution at the secondary site of CD is well-known in non-peptidic systems [30,31] and there are cases where secondary site modification was proved to be advantageous over the primary one [32,33]. Future studies that will compare the interactions taking place in molecule **3** to other molecules bearing the same peptide but attached through the primary site of the CD and/or longer spacers are expected to give a better understanding of the complexation process. Also, as mentioned before, information on the preferable conformation of the synthesized molecule **3**, as well as on future structural variants of it, will be of great value for a successful design and synthesis of conjugated LHRH analogues with higher therapeutic index.

3.2. Synthesis

The synthesis of the target molecule **3** was realized via coupling of 3-monoamino-β-CD to the modified with the εAhx spacer protected analogue of LHRH followed by removal in one step of all the protective groups (Scheme 1).

Thus, initially the synthesis of the analogue was performed by the Fmoc/*t*Bu methodology, utilizing the 2-chlorotrityl chloride (CLTR-Cl) resin [34–39]. The side chains of the amino acids were protected as follows: trityl group (Trt) for His, 2,2,4,6,7-pentamethyldihydrobenzofurane group (Pbf) for Arg and *tert*-butyl group (*t*Bu) for Ser and Tyr. The protected peptide was cleaved from the resin using the cleavage mixture CH_2Cl_2/TFE (7/3). The use of the CLTR-Cl resin and mild cleaving conditions allowed peptide release from the resin as well as the conservation of the side chain protecting groups. The purity of protected peptide was ≥98% as it was found by analytical RP-HPLC and the peptide was used without further purification in the coupling with β-CD.

In the next step, the protected peptide was coupled with 3-monoamino-β-CD, in liquid phase. DMF was used as solvent since the 3-monoamino-β-CD is adequately soluble only in water and highly polar solvents such as DMF and DMSO. In the coupling step, a reaction took place between the amino group of 3-monoamino-β-CD and the activated carboxylic acid (hydroxybenzotriazole derivative). There are various reagents for the activation of the carboxylic group. NHS or HOBt with DCC or DIC are among the usual ones while BOP, PyBOP, HBTU and TBTU represent more recent reagents [40–42]. These reagent mixtures with carboxylic groups give activated esters that are significantly more reactive towards amines than alcohols. This property is of crucial importance in our case since the ratio of amino groups to hydroxyl groups is 1 to 20. Three different systems, NHS/DCC, HOBt/DCC and TBTU/DIEA were tested (results not included). The best results were obtained with the HOBt/DCC system. The reactions were followed by TLC (*i*PrOH/H_2O 7:3) and ESI-MS [43]. CDs and amino-CD derivatives

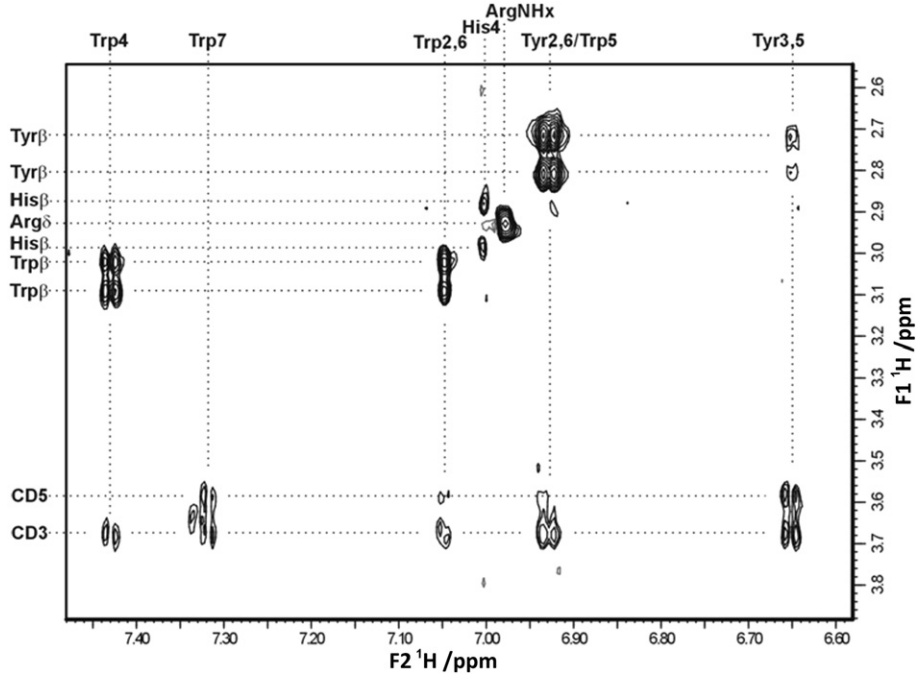


Fig. 2. Expanded region of NOESY spectrum of 2 mM conjugated peptide with β-CD **3** obtained at 298 K with mixing time of 75 ms in which the NOE connectivities between the peptide and β-CD segments are observed.

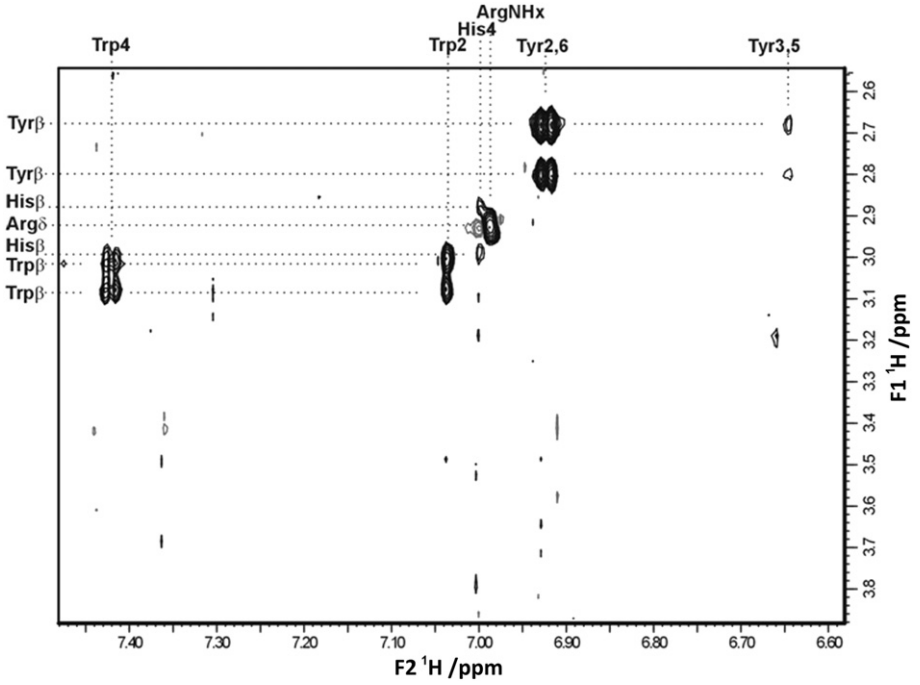


Fig. 3. Expanded region of NOESY spectrum of a mixture of peptide **2** (2 mM) with β-CD (2 mM) obtained at 298 K with mixing time of 75 ms. The same region is presented in Fig. 2.

are very polar compounds, and so eluents with very high polarity are necessary for the TLC on silica. The choice of *i*PrOH was based on the fact that solubility of β-CD is higher in mixtures of H_2O/*i*PrOH than in mixtures of water with other alcohols [43]. R_f values of 0.05 and 0.81 were found for 3-monoamino-β-CD and protected conjugate respectively. The product was isolated via precipitation with ether.

In the last step all protective groups present in the peptide unit were removed. These groups were a) Trt in the imidazole residue, b) two *t*Bu groups at the tyrosine and serine residues and c) Pbf at the arginine residue. Trituration with a mixture of 90% TFA in dichloromethane in the presence of 0.3% triethylsilane, anisole and H_2O is a well known system for the simultaneous removal of the above protective groups and was successfully applied in the case of peptide **1**. However, to obtain conjugate **3** a less drastic and anhydrous system was used (50% TFA in CH_2Cl_2 solution in the presence of 0.3% triethylsilane and anisole) in order to reduce the risk of hydrolysis of the CD moiety. Thus, deprotection in the final step of synthesis of conjugate **3** was successfully performed with the above anhydrous mixture within 4 h at RT. Isolation by precipitation with cold diethyl ether and purification with semi preparative RP-HPLC provided the final product as a white solid.

3.3. NMR conformational analysis

Fig. 1 shows the ^{1}H NMR spectra of the two molecules under study (peptide **2** and peptide–β-CD conjugate **3**).

The chemical shifts assignment of the protons (Supplementary Tables S1 and S2) and ^{13}C nuclei of the two molecules was achieved with the aid of the combined use of ^{1}H NMR, and 2D DQF-COSY, TOCSY, NOESY and ^{1}H–^{13}C HSQC spectra recorded in water/DMSO-d_6 solvent (9:1). This solvent provides an amphiphilic environment, mimicking the physiological conditions at the receptor binding site [44]. The proton–proton NOE connectivities were identified from

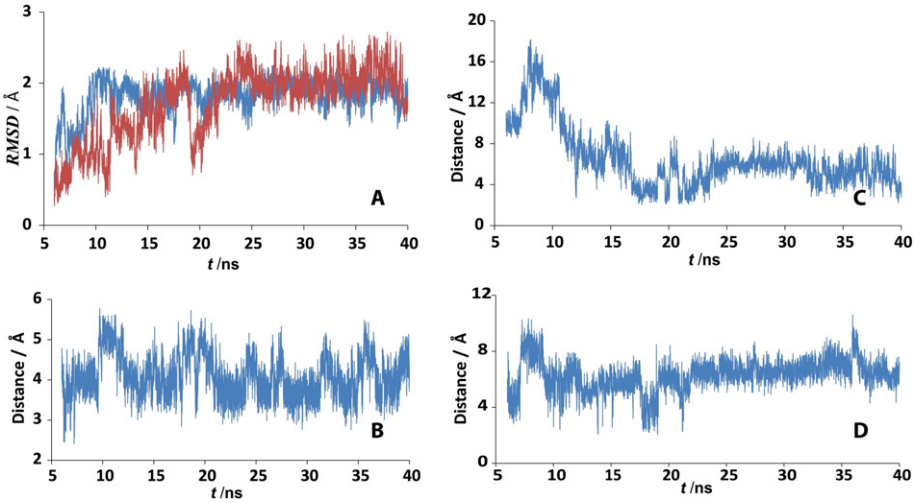


Fig. 4. (A) RMSD of the CD ring (blue), RMSD of the peptide backbone (red), (B) Distances of Tyr-H3 from CD-H3, (C) Distance of Trp-H7 with CD-H3 and (D) Distance of DLeu (H^{β}) from CD-H3 as they result from the MD simulation using Desmond.

NOESY spectra acquired with mixing times of 75 and 150 ms. Some spin-diffusion effects were observed at mixing time of 150 ms, therefore NOE connectivities recorded with mixing time of 75 ms were used for estimation of proton–proton distances.

Along the entire peptidic backbone in molecules **2** and **3** strong sequential NOE connectivities $d_{\alpha N}(i,i+1)$ are observed (Supplementary Tables S3 and S4), indicating a predominant population of extended backbone conformation for both molecules. A weak sequential $d_{NN}(i,i+1)$ is observed only between residues Gly^{10} and εAhx^{11}.

The ^{1}H NMR signals related to the CD part in β-CD (Supplementary Fig. S1a) and mixtures of β-CD with peptide were almost identical. Similarly, the ^{1}H NMR signals related to the CD part in 3-monoamino-β-CD (Supplementary Fig. S1b) and the conjugated peptide **3** (Fig. 1b) were almost identical. The assignment of their peaks was achieved using TOCSY and ^{1}H–^{13}C HSQC experiments.

On the other hand, the conformational similarity of the peptide chains in molecules **2** and **3** can be depicted by comparison of their ^{1}H chemical shifts (Supplementary Tables S1 and S2). Only at εAhx region, a significant chemical shift difference ($\Delta\delta = 0.1$ ppm) between the peptide and the conjugated peptide is observed. This is evidence that spacer in the case of conjugated peptide may show different conformational properties. Indeed, as it is pointed out in the Introduction, the spacer served as a conformational flexible tool in order the β-CD to gain capability to engulf various segments of the peptide.

In NOESY spectrum of 2 mM conjugated peptide recorded with 75 ms mixing time, clear NOEs between the internal H-3 and H-5 protons of β-CD (CD-H3, CD-H5) and the aromatic protons of Tyr and Trp are eminent (Fig. 2). These clear NOEs depict spatial vicinity of the interior lipophilic core of β-CD with the aromatic protons.

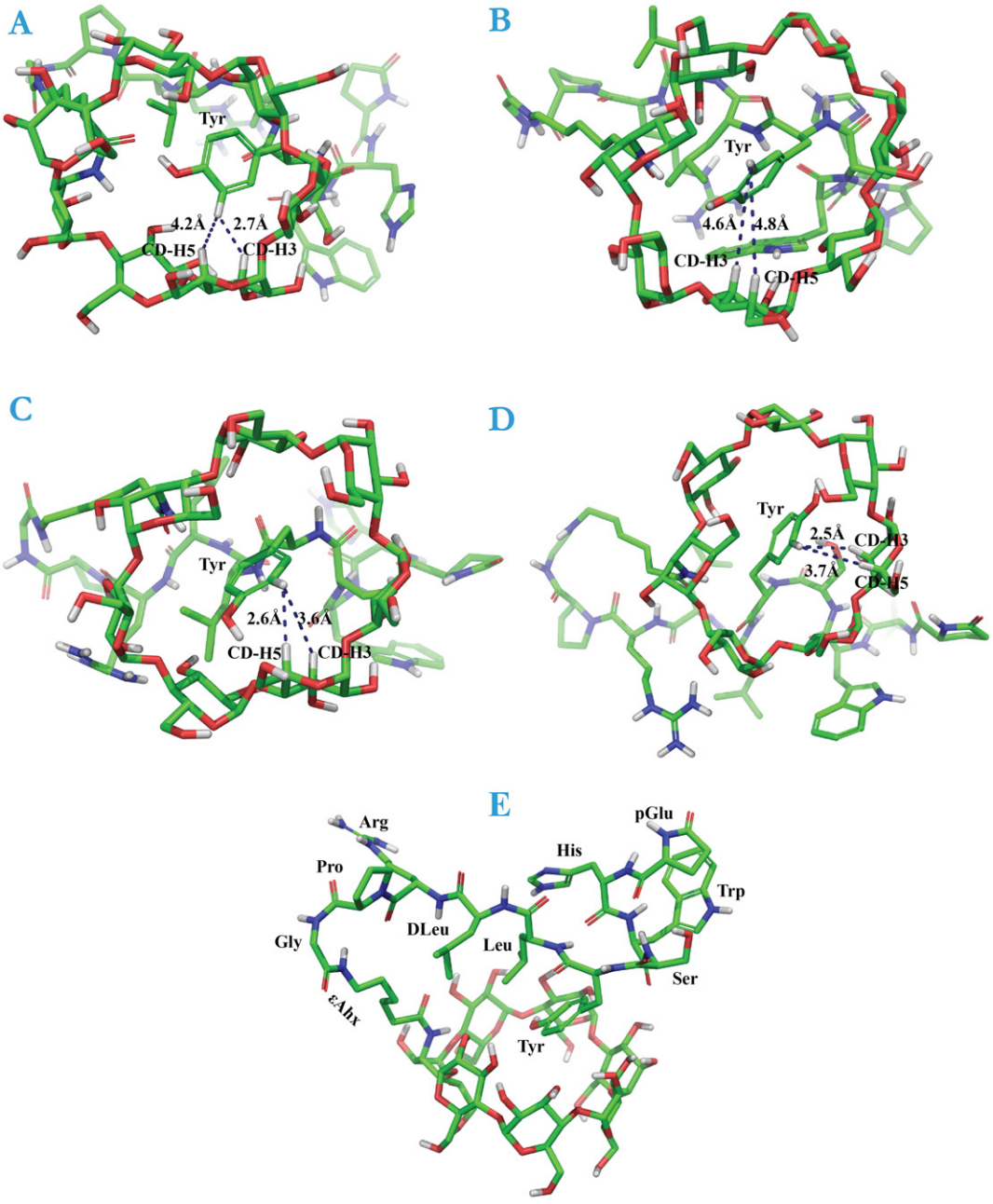


Fig. 5. Intermediate structures of the system during the MD simulation performed using MacroModel (constraints were applied on the tyrosine ring). (A) t = 50 ps, E = −397.050 kJ/mol, (B) t = 8.5 ns, E = −513.944 kJ/mol, (C) t = 17 ns, E = −429.219 kJ/mol, (D) t = 20 ns, E = −476.367 kJ/mol and (E) t = 12 ns, E = −573.435 kJ/mol (structure with the minimal energy).

There are two possibilities for the interpretation of these results: (a) intramolecular interactions exist between the peptide segment and β-CD aided by the spacer and (b) intermolecular interactions are formed owing to the vicinal approaching of two opposing segmental regions of the molecule.

To distinguish between the two possibilities, NOESY experiments were performed with mixtures of β-CD and peptide **2** at different concentrations and mixing times. Primarily, a NOESY experiment at 2 mM concentration for both β-CD and peptide **2** using 75 ms mixing time was performed. Since these conditions were identical to the ones used in the NOESY experiment of the conjugate peptide **3** (Fig. 2), a direct comparison of the NOE data can be performed. As it was found, no NOE cross-peaks between the peptide and the β-CD are observed (Fig. 3). Even at significantly longer mixing time of 500 ms only very weak intermolecular NOE between the aromatic protons of Tyr and the CD-H3 protons is observed (Supplementary Fig. S2).

From these results it can be concluded that there is no contribution of intermolecular interactions between the peptide and β-CD segments to the NOE cross-peaks of conjugated peptide **3** presented in Fig. 2. Only at higher concentrations, 10 mM of synthetic peptide and 10 mM of β-CD (ratio 1:1) and long-mixing time of 500 ms (referred to the literature as ideal to detect intermolecular interactions) clear NOEs are observed again between the internal protons CD-H3 and CD-H5 of β-CD and aromatic protons of Tyr and Trp (Supplementary Fig. S3) [45].

In molecule **3** the intramolecular NOE connectivities between the peptide and the β-CD units are very specific (Fig. 2). Only the aromatic rings of the Trp and Tyr are in close spatial proximity to the interior of β-CD. Thus, no NOEs are observed between the β protons of Trp (or Tyr) and protons of β-CD.

CD-H3 protons give NOE connectivities with both 2,6 (Tyr-H2,6) and 3,5 (Tyr-H3,5) protons of tyrosine, while the CD-H5 protons give NOE connectivities only with the Tyr-H3,5 protons. Similarly, in the case of

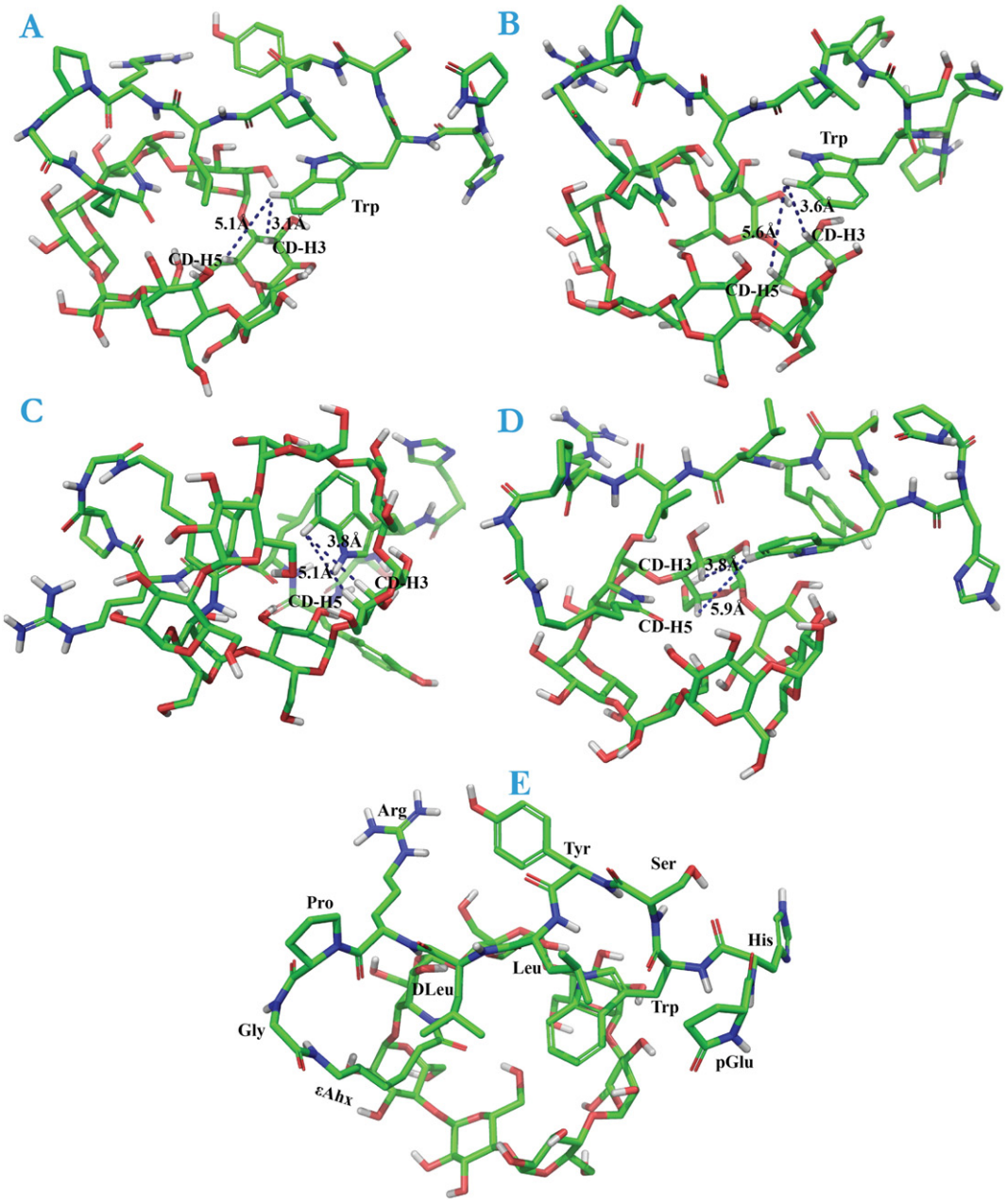


Fig. 6. Intermediate structures of the system during the MD simulation performed using MacroModel (constraints were applied on the tryptophan ring). (A) t = 50 ps, E = −500.927 kJ/mol, (B) t = 4.34 ns, E = −491.907 kJ/mol, (C) t = 8.68 ns, E = −566.139 kJ/mol, (D) t = 13.1 ns, E = −485.657 kJ/mol and (E) t = 14.02 ns, E = −625.774 kJ/mol (structure with the minimal energy).

tryptophan, proton 7 (Trp-H7) gives NOE connectivities with both the CD-H3 and CD-H5 while Trp-H4 and Trp-H6 protons give NOE connectivities only with CD-H3 protons. These specific spatial interactions indicate a very precise position of the aromatic rings in the interior of the β-CD. Therefore, the CD-H3 protons, located at the wide rim of β-CD, are found to be closer to the aromatic rings of Trp or Tyr compared to the CD-H5 protons, located at the narrow rim of β-CD.

To our knowledge the cavity of β-CD can host only one aromatic ring [46–49]. In our results the Trp and Tyr protons show inter-segment NOEs of similar intensity. Thus, it is evident that the εAhx spacer gives sufficient flexibility to conjugate **3** so that the β-CD can orient and engulf equally the two aromatic rings during the NOE build-up. To provide insight to the experimental results we proceeded with *in silico* MD calculations.

3.4. Molecular dynamics simulation

The potential energy, pressure, temperature and volume of conjugate **3** during the simulation time (40 ns) remained constant (results not included) indicating a stable system. The RMSD values of the CD ring and peptide backbone for the conjugate **3** are shown in Fig. 4A.

Special attention was paid during the simulation process for the aromatic rings of Tyr and Trp. According to the experimental NOEs the Tyr-H3,5 protons are in spatial proximity with the internal protons CD-H3 and CD-H5 of the CD segment. An extended conformation of the backbone of the peptide was manually constructed and constraints between Tyr-H3,5 and CD-H3 and CD-H5 were applied. As it is already stated, the simulation results showed that the system was very stable and an average distance between Tyr-H3,5 and CD-H3 of 3.23 Å was observed (Fig. 4B) while the average distance between Tyr-H3,5 and CD-H5 was calculated to be 4.83 Å. The system kept the extended form of the backbone in accordance with the absence of any medium or long range NOEs between the amino acids of the peptide. Additional average distances between Tyr-H3 and CD-H5, Tyr-H2 and CD-H5, Tyr-H2 and CD-H3, Trp-H4 and CD-H5 and between Trp-H6 and CD-H5 are shown in Fig. S4 (see supplementary).

The same methodology was applied with the aromatic amino acid of tryptophan (Fig. 4C). The derived trajectory in MD simulation showed an average distance between Trp-H7 and CD-H3 of 4.36 Å. The average distance between Trp-H4 and CD-H5 was 6.44 Å which justifies the absence of NOE correlation between the two protons (Fig. 4D).

In conclusion, these two semi-manually constructed models in which either Tyr or Trp is embedded in the interior of β-CD are shown through MD calculation to be stable and justify the NOE experimental results. Fig. 5 shows various snapshots of the trajectory of the conjugate peptide **3** during the simulation process as they are clustered in which Tyr is embedded in the CD core. The different low energy representative conformers depict clearly that Tyr is favorably situated in the core of the CD segment and in the vicinity of CD-H3 and CD-H5 protons. Fig. 6 shows various snapshots of the trajectory of the conjugate peptide during the simulation process as they are clustered in which Trp is embedded in the CD cavity. The different low energy representative conformers depict clearly that Trp is not as favorably situated in the core of the CD segment as Tyr and is in the vicinity of only CD-H3 proton. Thus, the two aromatic rings show distinct localization and orientation with the CD core, in a harmony with experimental NOE results. The distances between DLeu (H^{β}) from CD-H3 are shown in Fig. 4D. The obtained results were confirmed using Desmond MD calculations.

4. Conclusions

An LHRH analogue elongated with εAhx serving as a flexible spacer and conjugated with 3-monoamino-β-CD was successfully synthesized for the first time. The conformational properties of this molecule were studied using a combination of 2D NOESY experiments and MD simulations. The results obtained clearly show that aromatic segments of Tyr and Trp can approach with a different affinity the interior core of CD. The encapsulation of the two aromatic segments into the CD cavity might favor the drug delivery of the conjugate. It is of particular importance that spacer εAhx allows a dynamic conformational equilibrium which exchanges the entrance of the two aromatic rings in hydrophobic core of CD. The significance of the spacer to induce intramolecular interactions which might enhance the drug delivery will be further investigated. Thus, different conjugates are in the process of synthesis in which the spacer varies in length.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.10.017.

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