University of South Bohemia - České Budějovice
Faculty of Science
Department of Chemistry and Biochemistry

Bachelor Thesis

Synthesis and separation of novel bombesin analogues for targeted molecular radionuclide imaging and therapy in oncology

Jana Beranová

May, 2012

Supervisor: Ing. Martin Kropáček, PhD.
Radiopharmaceutical Department
Nuclear Physics Institute of the ASCR,v.v.i.
Husinec - Řež

External Supervisor: prof.RNDr. Libor Grubhofer, CSc.
Faculty of Science
University of South Bohemia
České Budějovice

Bachelor Thesis:

Beranová, J., 2012: Synthesis and separation of novel bombesin analogues for targeted molecular radionuclide imaging and therapy in oncology. BSc. Thesis in English – 26 p. Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation:

Several human tumors including prostate cancer express massively increased levels of Gastrin Releasing Peptide receptor and its subtypes (NMB-R and BB3-R). Peptide bombesin, which has an ability to bind to all three mentioned receptors, has drawn an attention of many researchers. The developement of bombesin conjugated radiopharmaceuticals as tracers for targeted nuclear imaging and as therapeutical drugs represents a promising approach in cancer treatment.

Simple synthesis and separation of novel conjugates of [Lys_3] bombesin with macrocyclic chelators DOTA, NOTA and PCTA is described in this work. The formation of stable NOTA and PCTA complexes with radiometals (^{177}Lu and ^{111}In) proceeds very fast within 10 - 15 minutes.

Affirmation:

I hereby declare that I have worked on my bachelor's thesis independently and used only the sources listed in the bibliography.

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full form to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages.

Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagerism detection system.

České Budějovice, 14. 5. 2012 Signature.....

Jana Beranová

Acknowledgements:

I would like to acknowledge the management of the Nuclear Physics Institute of AS CR for giving me an opportunity to participate in their research. My thanks go to my supervisors who provided me with professional guidance and kindly shared their experience.

Special thanks belong to my family and friends who supported me along the way.

The research was made possible thanks to:

EUREKA Project E!3832 http://www.eurekanetwork.org/project/-/id/3832
"Radionuclide precursors and radiopharmaceuticals for targeted radionuclide imaging and therapy in nuclear medicine"
Czech Part EUREKA OE08018, Czech Ministry of Education,Youth and Sports
Principal Investigator and Coordinator of the Project:
Ing. Miloš Beran, CSc.
Nuclear Physics Institute of AS CR, public research institution
Husinec – Řež

Table of Contents:

List of Abbreviations:

BBN.....Bombesin

GRP-R.....Gastrin Releasing Peptide - Receptor

NMB-R.....Neuromedin B - Receptor

PET.....Positron Emission Tomography

SPECT.....Single Photon Emission Computed Tomography

BFC.....Bifunctional Chelate

EDTA.....D**iethylenetriaminepentaacetic Acid**

DTPA.....1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic Acid

DOTA.....1,4,7,10- tetraazacyclododecane-1,4,7,10-tetraacetic Acid

TETA.....1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic Acid

NOTA.....1,4,7-triazacyclononane-1,4,7-triacetic Acid

PCTA.....3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-triene-3,6,9-triacetic Acid

SPPS.....Solid Phase Peptide Synthesis

Fmoc.....9-fluorenylmethyloxycarbonyl

Aca.....ε-Aminocaproic Acid

SCN.....Isothiocyanate (rhodanide) group

TRIS.....2-Amino-2-hydroxymethyl-propane-1,3-diol

HPLC.....High Performance Liquid Chromatography

TFA.....Trifluoroacetic Acid

1. INTRODUCTION

1.1. Background

Radiopharmacy is an important field of medicine which deals with development and production of radioactive drugs. Molecular targeting in oncology, one of the rapidly developing branches of nuclear medicine, represents a very promising approach in diagnostics and therapy of various health disorders. Radiolabeled molecules possess a great potential as tracers for metabolic pathways and organ function *in vivo*. Development of drugs for cancer, a worldwide leading cause of death [1] has become a main interest of many research programs.

The aim of the cancer drug investigation is to develop pharmaceuticals which can effectively help in diagnosis and treatment of the diseased tumorous tissue and at the same time to reduce the harmful effects of drugs on the healthy one.

1.2. Molecular targeting

The basic idea behind the molecular targeting lies in cell signaling principle. A great knowledge of biochemical pathways provides us with a chance to follow or even shut down a particular pathway without disturbing the others. Cell signaling, which governs the basic activities on cellular level, is based on the key and lock principle.

The protein molecules embedded in a plasma membrane of cell function as receptors to which a specific signaling molecules (=Ligands) can bind. Ligand is usually a small peptide, monoclonal antibody or other molecule (hormone, protein, toxin etc.). The receptors are very specific in shape and therefore they only allow particular signaling molecule to attach. If the proper ligand is „inserted“, a specific biochemical pathway is commanded.

Monoclonal antibodies were first considered as potential agents for targeting specific antigens. The results of the research led to further development of effective radiopharmaceuticals for radioimmunoimaging and radioimmonotherapy. Nowadays, one of the most promising approaches in nuclear molecular medicine is a use of peptide/receptor system.

1.3. Peptide/Receptor System

The ability of peptide molecules to uniquely bind to its specific receptors gives us a chance to develop new drugs which are directly targeted to malignant tumors while reducing harmful effect to normal tissues.

Several human tumors overexpress characteristic receptors which therefore become potential targets for radiolabeled peptide molecules. The development of peptide/receptor based radiopharmaceuticals represents a promising future for targeted nuclear imaging (*in vivo* visualization) and therapy. The aim in development of radiolabeled peptides is to synthesize a drug which is effective and at the same time less invasive than current methods such as chemotherapy and radiotherapy. One of such biomolecules with a great potential to become an effective targeted carrier is a 14 aminoacid peptide bombesin (BBN).

1.4. Bombesin and Bombesin-like Peptides

The original discovery of bombesin can be traced back to 1971. Anastasi et al. [2] first isolated and characterized the tetradecapeptide (sequence of aminoacids Glu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2) from skin of European frog *Bombina Bombina*. The amphibian peptide (**Fig. 1**) was found to be responsible for many physiological processes including smooth muscle contractions.

Further research concerning bombesin and closely related bombesin-like peptides soon led to a discovery of mammalian BBN analogue with strong structural homology to the amphibian one. McDonald et al. demonstrated that BBN-like peptides are widely distributed in mammalian tissues, where they play an important role in various biochemical processes.

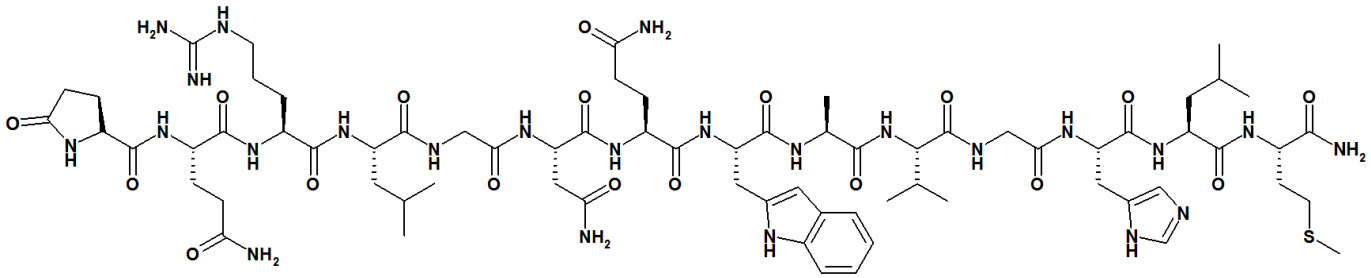


Figure 1: *Schematic structural formula of bombesin*

The first mammalian BBN homologue characterized by McDonald et al. [3] so called Gastrin Releasing Peptide (GRP) is a decapeptide consisting of 27 amino-acid structures with only histidine/glutamine substitution on position 8 from the carboxyl terminal. Nowadays, two different mammalian BBN-like peptides are known. The first being already mentioned GRP, which is responsible for stimulation of release of gastrine (as well as other gastrointestinal hormones) and the second one being Neuromedin B (NMB), whose exact function has not been established yet.

The medical research during past decades have shown that several human tumors massively overexpress GRP receptors (GRPRs). The increased levels of bombesin-like peptide receptors were found especially in prostate and breast cancer tumors. The unique ability of BBN to bind to the receptors present in cancerous cells of GRP-positive tumors gives the peptide a great potential to become a drug used not only for targeted nuclear imaging but also for therapeutic purposes and cancer treatment itself.

1.5. Bombesin and its Receptors

There are three known BBN receptors present in mammalian tissue. The GRP-R with high affinity to GRP, NMB-R with high affinity to NMB and also an orphan receptor BB3, for which corresponding ligand has not been discovered yet. [2] All three above mentioned receptors belong to a G-protein group, which consist of receptors located at the outer membrane of cells.

The amphibian bombesin expresses high affinity to all three receptor subtypes and therefore represents an ideal targeted biomolecule for treatment of GRP-R positive tumors such as prostate cancer.

1.6. Prostate Cancer

Prostate cancer is one of the most common types of malignant tumors in man with a high death rate worldwide. However, when diagnosed in its early stages, the cancer survival rate dramatically increases. The development of new sensitive and efficient diagnostic drugs is therefore very important.

Applying the molecular targeting principles in positron emission tomography (PET) has appeared to be a very successful way to localize and visualize prostate cancer cells *in vivo*. Different bombesin cojugates have been developed as potential targeted carriers of radiotracers for both PET and SPECT (single photon emission computed tomography). Many imperfections, such as high uptake of the radiopharmaceuticals by healthy tissues or instability of chelator biomolecules *in vivo*, lead to further research and development of BBN/GRP-R based targeted radiopharmaceuticals. Investigation of radiopharmaceuticals targeted to GRP-R receptors, which are overexpressed in the prostate cancer tumors, has become essential for diagnosis and treatment of the disease.

1.7. Radionuclide Imaging and Therapy

Due to known metabolic pathways of certain malignant tumors there is a possibility to detect the diseased tissue by the specifically targeted biomolecules. The use of radiolabeled bombesin (among other peptides) represents a suitable method for targeted nuclear imaging. The labeled BBN conjugates can be used as tracers for PET and SPECT. Both techniques represent minimally invasive methods of visualization *in vivo*. Small amounts of radioactive isotopes incorporated in the biomolecule carriers are injected into blood and used as tracers for cellular changes. The radioactive biomolecules bind to the diseased tissue with high affinity whereas the uptake in normal tissues is minimal, therefore it is possible to visualize tumor and it´s metastases in most cases.

Commonly used radiometals in diagnostic radiopharmaceuticals for PET include ^{64}Cu, ^{61}Cu, ^{68}Ga and ^{94m}Tc (positron emitting isotopes). In SPECT the most often used are ^{99m}Tc, ^{67}Ga and ^{111}In radionuclides (photon emitting isotopes). Radiolantanides ^{90}Y and ^{177}Lu and copper radinuclide ^{67}Cu (beta emitting radionuclides) are considered to be most suitable for therapeutic use.

1.8. Radiolabeled Peptides

The aim of the research is to develop a metabolicaly resilient radiolabeled peptide which will acumulate in target tissue and at the same time will be minimally absorbed by the healthy one. The development of radiolabeled targeted drugs is however accompanied by many obstacles. The choice of a suitable carrier, chelating agent, crosslinker and also a radionuclide determines the character and behavior of the drug *in vivo* and therefore is essential for its succesful synthesis.

The research focuses on developement of a targeted labeled peptide molecule, which posesses it´s original biological activity even after conjugation and labeling, is stable *in vivo*, is maximally uptaken by the target tissue and also excreted via renal system instead of the hepatobiliar/gastrointestinal tract. A developement of such a drug is not an easy task. However, promising results from recent studies lead to further developement of various radiolabeled BBN conjugates which have potential to be used as peptide/receptor based diagnostic and therapeutic drugs.

The choice of the radiometal used for labeling of the conjugated biomolecule is highly dependent on the application of the drug being developed. The character of the radionuclides (such as its half-life, type of radiation ect.) determines its choice for labeling.

1.9. Bifunctional Chelators for Conjugation with Biomolecules

Another essential factor in radiolabeling of biomolecules is a choice of bifunctional chelators (BFCs). They should serve as a stable and firm coupling between radiometal and biomolecule. There are many types of BFCs of various nature. The choice of suitable bifunctional chelator is highly dependent on the character of radiometal. The chelator molecule must have a strong coordination to a radionuclide which is used for the construction of a radiophamaceutical and most importantly it has to possess an ability to form a stable radiometal chelate complex.

However not only the choice of chelating agent itself determines the behavior of radiopharmaceutical. The scientific research have shown, that conjugation of BFC directly to the peptide molecules can cause a great decrease of binding activity of the molecule. To avoid this, the behavior of the radiolabeled conjugated biomolecule is modified by insertion of various spacers which are introduced between the chelator and biomolecule. Their nature varies from simple carbon chains to more elaborate molecules. The conjugation group used for attachment of the BFC to a biomolecule has a great effect on the character of synthesized drug.

Among others, the linker is able to modify the excretion of the radiopharmaceutical, increase a lipophilicity of biomolecule and to effect its biodistribution in the body. To develop a drug suitable for targeted cancer therapy and diagnosis, we have to find a perfect combination of all above mentioned parameters.

When designing new BBN derivatives, it is important to maintain the key sequence of 8 amino acids adjoining to the terminal aminogroup (**Fig. 2**).

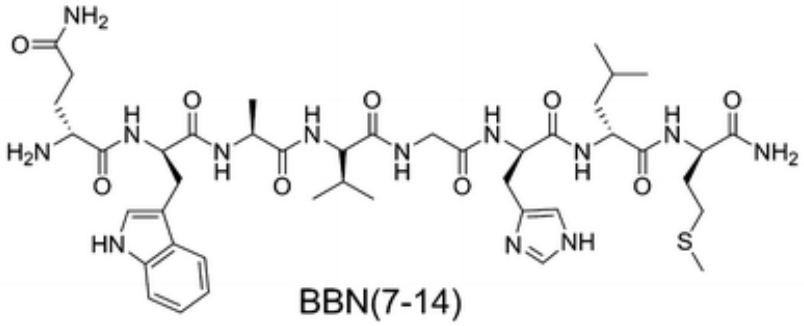


Figure 2: *Structural formula of truncated BBN - sequence of 8 key amino acids*

BFCs can be attached to this basic structure via various spacer chains. Many chelatation groups like ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid

(DTPA), 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA) and others [4-7] have been used for conjugation with bombesin. Nowadays 1,4,7,10- tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) and its bifunctional derivatives are the most commonly used macrocyclic chelators in preparation of BFC-BBN conjugates.

1.10. Most Important Radiolabeled DOTA-Bombesin Conjugates

Few conjugates of BBN with macrocylic chelators, including DOTA[Pro^1,Tyr^4]BBN and DOTA[ε-Lys^3,Tyr^4]BBN, were synthesized and labeled with ^{111}In by Breeman et al. **[**8**].** Among the synthesized radiolabeled structures, the ^{111}In-DOTA-[Pro^1,Tyr^4]BBN showed the highest uptake of radioactivity in GRP receptor-positive tissues as well as target-to-blood ratios. This conjugate labeled with ^{64}Cu or ^{86}Y was studied later by Biddlecombe et al. [9]. It`s schematic structure is shown in **Fig. 3**.

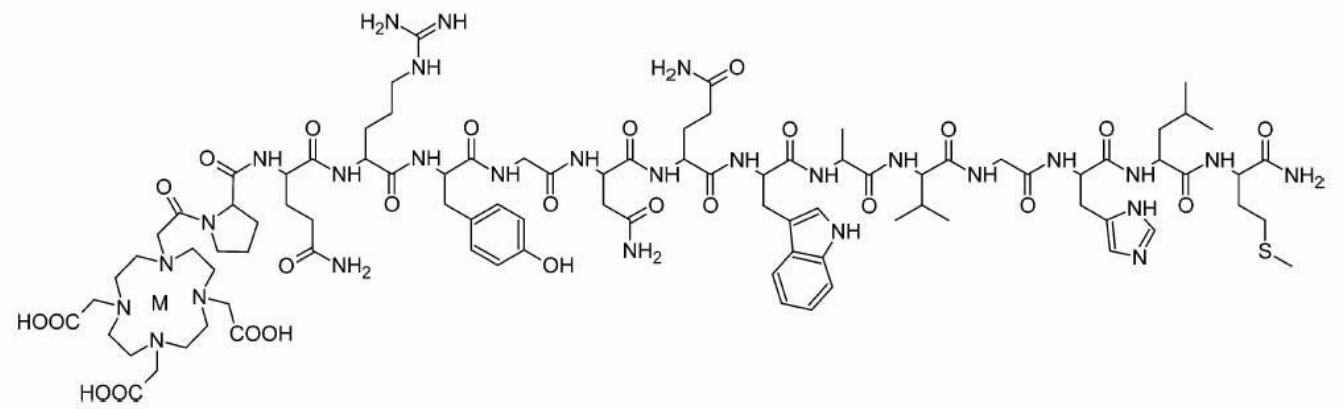


Figure 3: *Schematic structure of M-DOTA-[Pro^1,Tyr^4]BBN, M = ^{64}Cu or ^{86}Y*

Both synthesized radiopharmaceuticals for PET imaging of tumors with overexpressed GRP receptors are already included in the Molecular Imaging and Contrast Agent Database **[**10,11**].**

Another two DOTA-BBN conjugates labeled with ^{64}Cu, DOTA-[Lys^3]BBN and DOTA-Aca-BBN(7-14) (Aca = ε-aminocaproic acid, the carbon chain linker) were synthesized and tested in preclinical assays [12,13]. Considering the affinity to the GRP receptors, *in vivo* metabolic stability and tumor-to-background ratio, ^{64}Cu-DOTA-[Lys^3]BBN appears to be superior to the ^{64}Cu-DOTA-Aca-BBN(7-14). It`s structure is shown in **Fig. 4**.

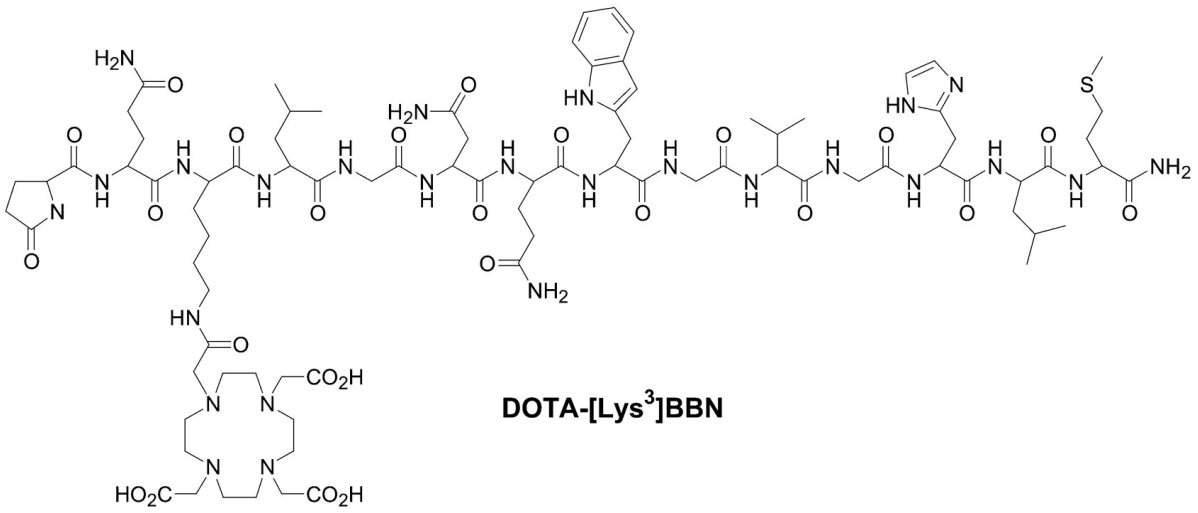


Figure 4: *Structure of DOTA-[Lys^3]Bombesin*

The most widely used chelator for conjugation with biomolecules and their radiometal labeling is DOTA. The main disadvantage of this macrocyclic chelator seems to be its lower metabolic stability - radiometals are often released from biomolecules *in vivo*. Further, the radiolabeling of DOTA conjugates usually requires heating of the reaction solutions nearly up to 100 °C. In some cases the high temperatures can cause significant changes in imunologic behaviour of bombesin analogues. Last but not least, the solid-phase peptide synthesis (SPPS) with Fmoc (9-fluorenylmethyloxycarbonyl) protecting group is ordinarily used to synthesize BBN analogues. This method does not belong to a commonly performed ones in radiopharmaceutical laboratory.

\

2. AIM

Two novel macrocyclic BFCs, *p*-SCN-Bn-PCTA and *p*-SCN-Bn-Oxo-DO3A, have been recently developed [14-17] and became commercially available (**Fig. 5**). The main advantage of the PCTA chelate is fast complexing of radiometals with no need to heat the reaction solutions. We decided to conjugate *p*-SCN-Bn-DOTA, *p*-SCN-Bn-NOTA and *p*-SCN-Bn-PCTA with [Lys^3]Bombesin, which is nowadays commercially available as well. The conjugation takes place in a solution via reaction of BFC's rhodanide group with lysine amino group in the lateral spacer chain of [Lys^3]BBN. The novelty of BFC-[Lys^3]BBN conjugates being prepared lies in the direct linkage of the spacer to one of the core carbons located on the macrocycle. In the previously synthesized BFC-[Lys^3]BBN (Fig. 4), one of macrocycle carboxylic groups is used for attaching instead. This may have an influence on complexation properties of macrocyclic chelators and subsequently on the behavior of the radiopharmaceuticals in general.

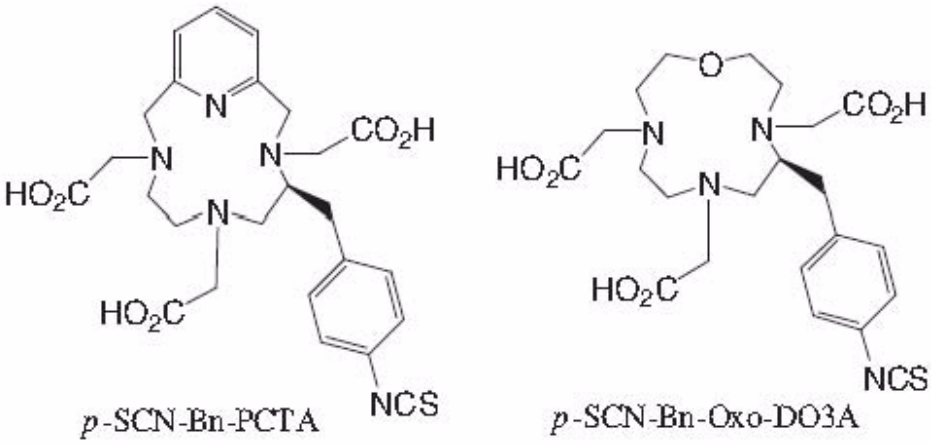


Figure 5: *Structure of the bifunction chelates p-SCN-Bn-PCTA and p-SCN-Bn-Oxo-DO3A*

3. EXPERIMENTAL PART

3.1. Materials

- [Lys^3]Bombesin (Glp-Gln-Lys-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH2, $C_{71}H_{110}N_{22}O_{18}S_1$, MW=1591.9) was purchased from American Peptide (Sunnyvale, CA, USA) and AnaSpec (Fremont, CA, USA) as a white lyophilized substance that is long-term stable when stored below -20 °C.
- Bifunctional macrocyclic chelating agents, *S*-2-(4-isothiocyanatobenzyl)-1,4,7,10-tetra-azacyclododecane-N,N',N'',N'''-tetraacetic acid (*p*-SCN-Bn-DOTA), *S*-2-(4-isothiocyanato-benzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (*p*-SCN-Bn-NOTA) and *S*-2-(4-isothio-cyanatobenzyl)-3,6,9,15-tetraazabicyclo[9.3.1]pentadeca-1(15),11,13-triene-3,6,9-triacetic acid (*p*-SCN-Bn-PCTA), were acquired from Macrocyclics (Dallas, TX, USA).
- Reaction solutions were buffered using Ammonium acetate, SigmaUltra, > 98 % (Sigma-Aldrich, Steinheim, Germany), Sodium dihydrogen phosphate Monohydrate and di-Sodium hydrogen phosphate Dodecahydrate (Fluka, Sigma-Aldrich) or TRIS (2-Amino-2-hydroxy-methyl-propane-1,3-diol) Suprapur Quality, > 99.9 % (Sigma-Aldrich). Their pH was adjusted using 30 % hydrochloric acid, Suprapur (Merck, Darmstadt, Germany) or 50 % solution of sodium hydroxide, p.a. (Merck).
- Ultrapure water (Aquatec Water Systems, CA, USA) degassed by boiling and subsequent cooling under nitrogen flow was used for all the procedures. Acetonitrile, HPLC gradient grade, > 99.9 % (Merck) and Trifluoroacetic acid (TFA) puriss. p.a., for HPLC (Sigma-Aldrich) were used for HPLC gradient elution.

3.2. Instrumentation and Methods

- ***Incubation of reaction solutions*** at constant temperature (commonly at 4 °C) in polypropylene Eppendorf microtubes 3810X, volume 1.5 ml (Hamburg, Germany) was performed in the thermostated incubator BIOSAN CH-100 (Riga, Latvia). Micropipets Eppendorf (Hamburg, Germany) and Biohit (Helsinki, Finland) were used for handling very small volumes (usually up to 1 ml) of liquids.

- For ***chromatographic characterization and isolation*** of prepared products the Agilent 1200 Series HPLC System equipped with Degasser G1322A, Quaternary

Gradient Pump G1311A,Variable Wavelength UV/VIS Detector G1314B adjusted at 254 nm (Agilent, Waldbronn, Germany) and Rheodyne Injector (Milford, USA) were employed. The Gina Star version 4.07 software (Straubenhardt, Germany) was used for data processing.

- Reversed phase GRACE Vydac 218MS54 (C18) *analytical column* with 4.8 mm ID and 250 mm lenght, particle size 5 μm (Columbia, MD, USA) and 20 μl injection loop was employed for monitoring reaction components during development of methods for syntheses of BFC-[Lys^3]BBN conjugates. The eluent flow was 2 ml/min, with the mobile phase starting from 95 % solvent A (0.1 % TFA, in water) and 5 % solvent B (0.1 % TFA in acetonitrile) to 35 % solvent A and 65 % solvent B at the end of separation (32 min).
- When optimal conditions for syntheses were found, reversed phase GRACE Vydac 218MS510 (C18) *semipreparative column* with 10 mm ID and 250 mm lenght, particle size 5 μm (Deerfield, IL, USA) and 100 μl injection loop was used for isolation of products in amounts needed for further application. The eluent flow was 3 ml/min, with linear gradient of the mobile phase similar to that used for analytical column. (summarized in **table 1)**:

Table 1*: HPLC*

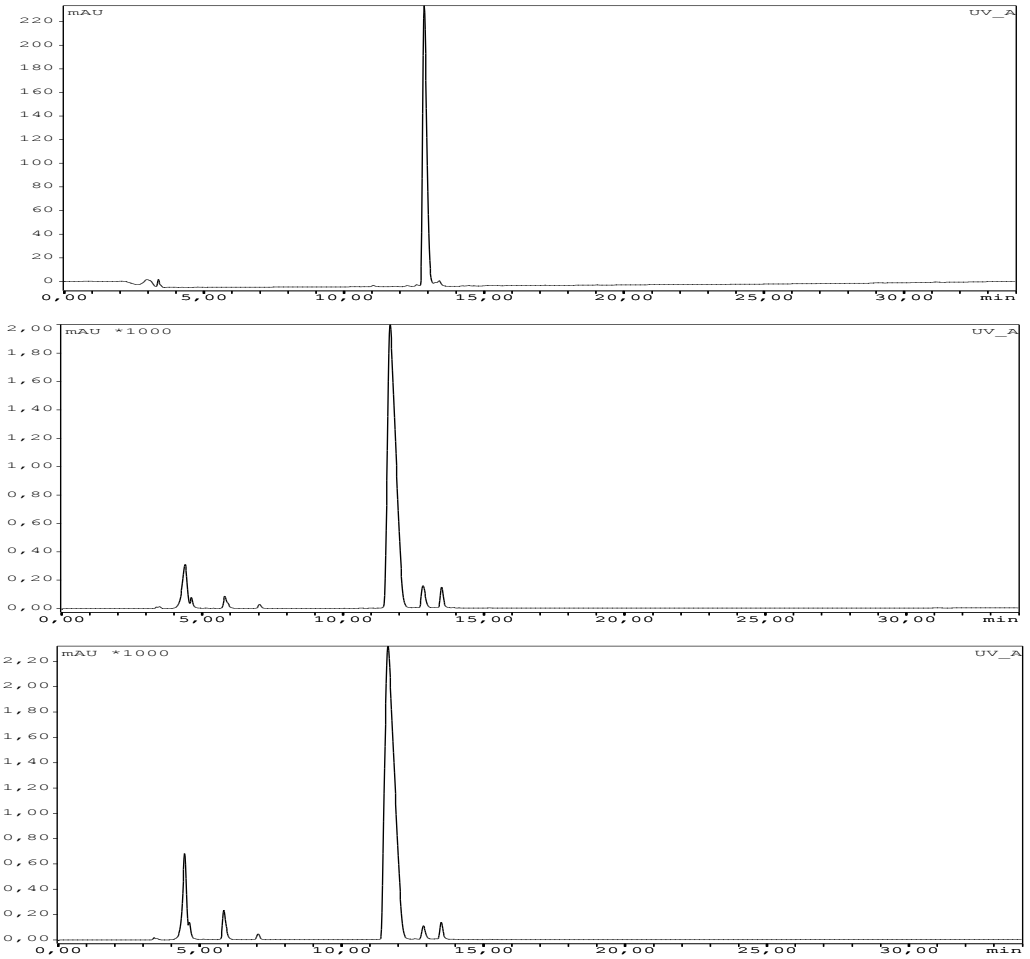
Step No.	**Time of separation (minutes)**	**% A (0.1 % TFA in water)**	**% B (0.1 % TFA in acetonitrile)**
1	0	95	5
2	2	95	5
3	32	35	65
4	34	35	65

- Chromatographic fractions containing BFC-[Lys^3]BBN conjugates were collected (size of fraction about 2 - 3 ml) in 10 ml glass vials and lyophilized in LABCONCO (Kansas City, MO, USA) vacuum freeze drier. The samples were deeply frozen in liquid nitrogen before lyophilization in order to avoid their foaming and leakage from vials due to high content of volatile acetonitrile. The products were stored at - 20 ºC.

4. RESULTS

Conjugation reaction of [Lys^3]Bombesin with isothiocyanate (rhodanide) functional group of macrocyclic bifunctional chelate takes place in slightly alkalic water media (pH 8 - 9). The reaction is based on addition of CNS group to lysine amino group located in the lateral carbon chain of the peptide, thus forming the -NH-CS-NH- coupling article between the two molecules. However, isothiocyanate group of the BFC is undergoing fast hydrolysis under these conditions. That is why large stoichiometric excess of BFC over [Lys^3]Bombesin has to be used in the reaction. This molar excess was usually about 4 : 1 in our work.

p-SCN-Bn-DOTA bifunctional chelate was used to investigate conditions for synthesis of its conjugate with [Lys^3]Bombesin. This BFC is quite cheap and DOTA is most common among macrocyclic chelators. 1.3 mg of [Lys^3]Bombesin were first dissolved in 0.5 ml of 0.5 M ammonium acetate with pH adjusted to 8.9. 2.2 mg of *p*-SCN-Bn-DOTA were subsequently added and dissolved by mild swirling. The solution was then placed into an incubator and thermostated to 4 °C. In specific time intervals, the samples were taken out and analyzed via HPLC using Vydac 218MS54 C18 analytical column. Results are shown in **Fig. 6.**



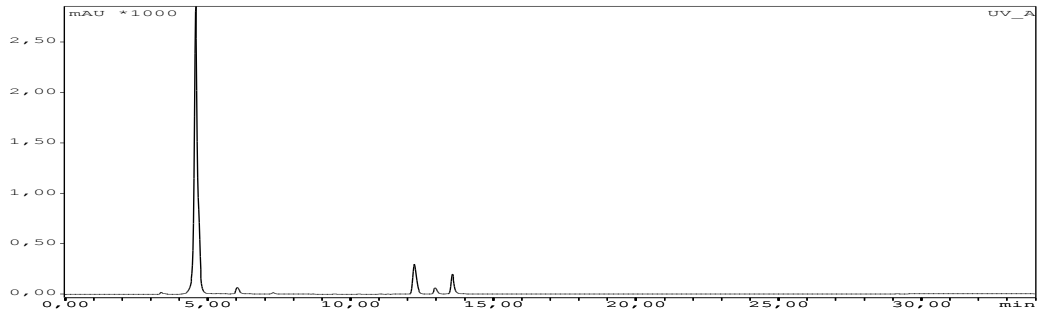
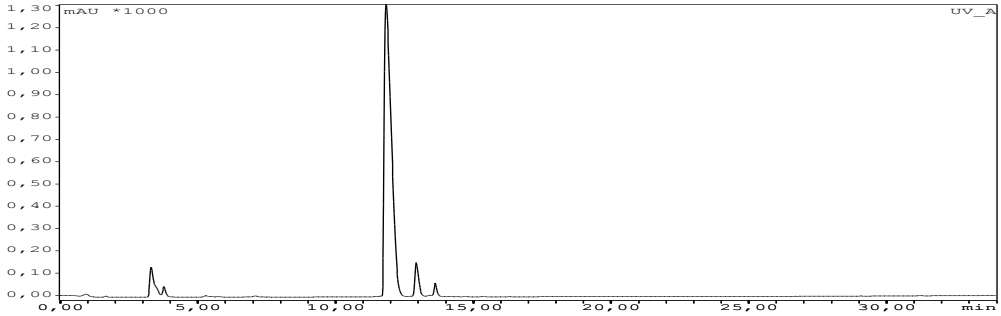


Figure 6: *HPLC analysis of the conjugation of [Lys^3]Bombesin with p-SCN-Bn-DOTA in 0.5 M ammonium acetate (pH = 8.9)*

- Chromatographs of the solution of [Lys^3]BBN and its mixture with p-SCN-Bn-DOTA incubated at 4 ºC for 1, 2 and 14 hours (from top to the bottom respectively)

The conjugation reaction was quite fast and the decomposition of the conjugate took place after its maximal concentration was reached. The product was nearly fully decomposed after 14 hours of incubation. Furthermore, the reaction wasn`t reproducible (niether in acetate or phosphate buffer) and was highly sensitive to polymerization of the peptide (gel formation) which was caused by vigorous stirring or rapid pH changes. The assumption drawn from the results is that the buffering capacity of the used buffers in the pH region of 8-9 is not sufficient for the reaction.

Therefore the 0.1 M TRIS buffer, which is known to be suitable for buffering between pH 8 and 9, was used instead. [Lys^3]Bombesin was easily dissolved in water, calculated volume of 1 M TRIS along with *p*-SCN-Bn-DOTA were added and pH was then adjusted to 8.5 . No further problems with peptide coagulation were observed and the decomposition of conjugate was much slower than in previous cases - see **Fig. 7**.



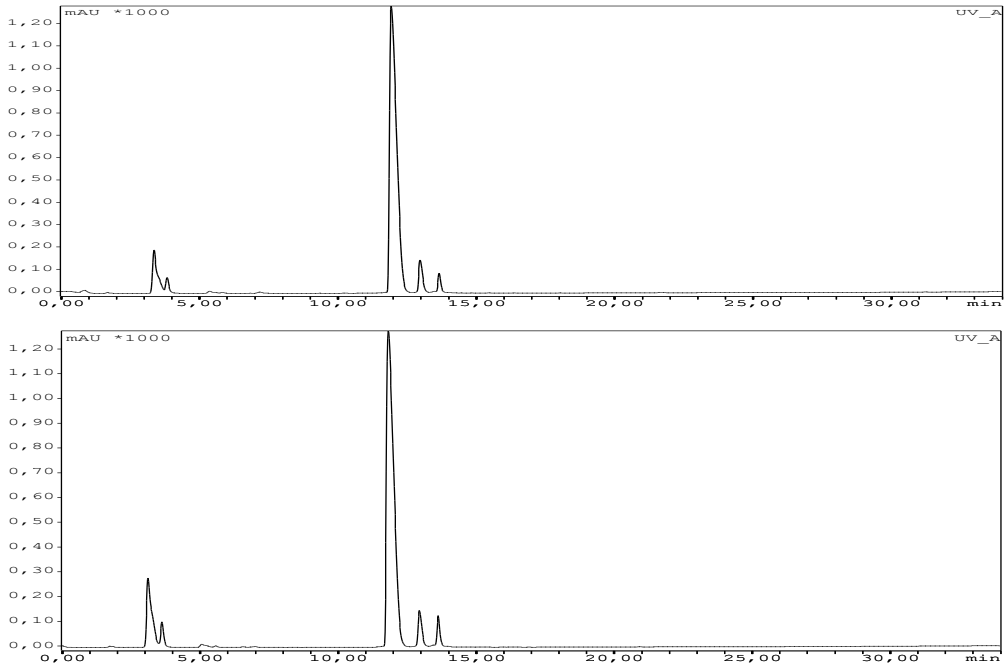
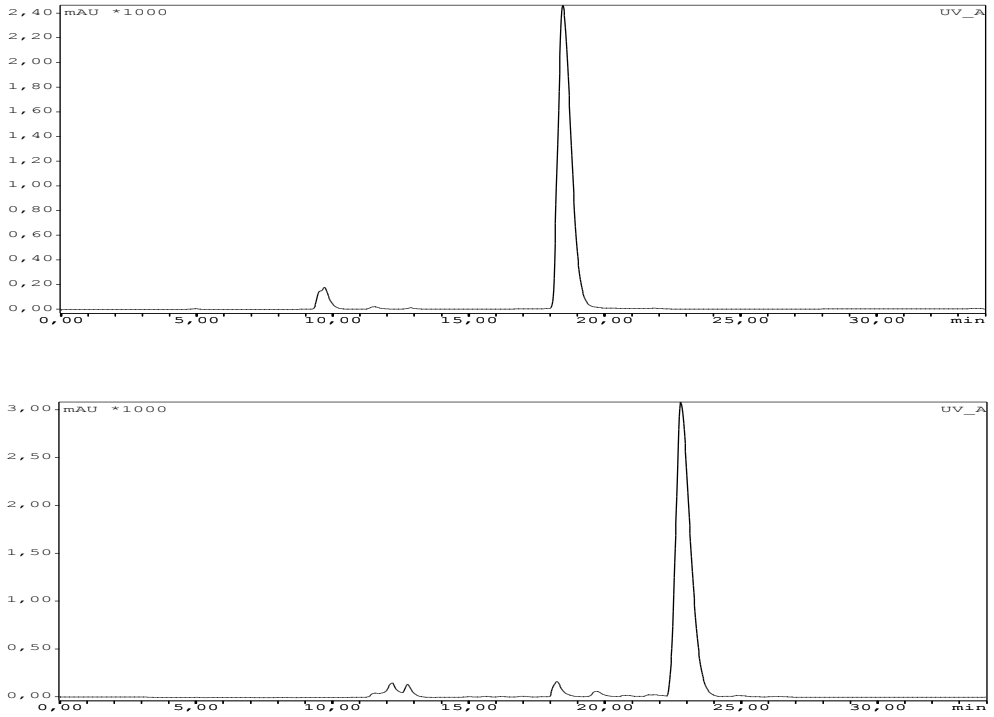


Figure 7: *HPLC analysis of the conjugation of [Lys^3]Bombesin with p-SCN-Bn-DOTA in 0.1 M TRIS buffer (pH = 8.5)*

- mixture of [Lys^3]BBN and p-SCN-Bn-DOTA incubated at 4 ºC for 1, 2 and 5 hours (from top down respectively)

According to the obtained results, following ***procedure for the preparation of conjugates*** was suggested:

- weigh in 1.3 mg of [Lys^3]Bombesin into a transparent glass vial (volume 2 ml) in order to easily observe it`s dissolution;
- add 0.5 ml of degassed water and disolve [Lys^3]BBN by cautious bubbling using a micropipette tip;
- add 10 µl of 1 M hydrochloric acid, 50 µl of 1 M TRIS and stirr;
- weigh 2.2 mg of BFC (*p*-SCN-Bn-DOTA, -NOTA or -PCTA) into a vial and gently stirr until fully disolved;
- transfer the resulting solution into 1.5 ml polypropylene conical Eppendorf tube and adjust the final pH to 8.5 with about 10 µl of 1 M TRIS;
- place the tube with the reaction mixture in a thermostated incubator at 4 °C;
- after 30 minutes of incubation inject 100 µl of the reaction solution into Vydac 218MS510 C18 semipreparative column and collect the product (conjugate) fraction manually (see **Fig. 8**) into 10 ml glass vial with lyophilization stopper.



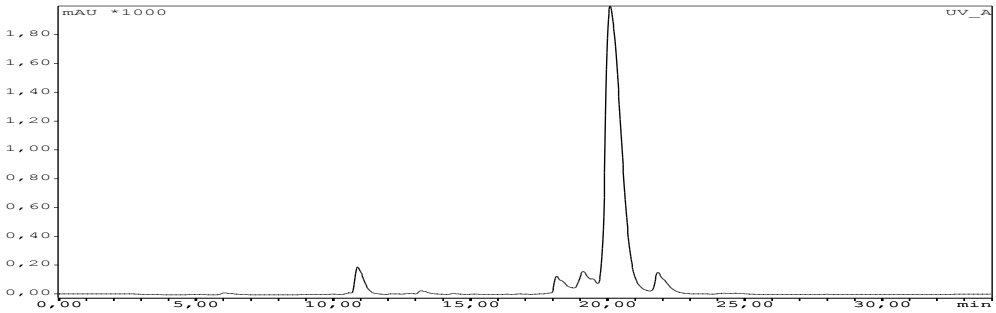


Figure 8: *Semipreparative HPLC isolation of BFC - [Lys^3]Bombesin conjugates - DOTA-p-Bz-NH-CS-NH-[Lys^3]BBN,NOTA-p-Bz-NH-CS-NH-[Lys^3]BBN and PCTA-p-Bz-NH-CS-NH-[Lys^3]BBN (form top down respectively)*

Decomposition (hydrolysis) of conjugates is very slow at pH = 8.5 and 4 °C - see **Fig. 7**.
It is therefore possible to process all the reaction solution (~ 0.5 ml) in 5 subsequent HPLC separations. The reaction is terminated during separation in acidic eluent (0.1 % TFA in water/acetonitrile mixture) and the resulting product is obtained as a stable BFC-[Lys^3]Bombesin trifluoroacetate salt. No further purification of products was performed.
The isolated conjugates were lyophilized after deep prefreezing in liquid nitrogen and stored at - 20 °C (~ 200 μg per vial) for next experiments.

5. DISCUSSION AND CONCLUSIONS

Schematic structure of the novel [Lys^3]Bombesin analogues with conjugation to macrocyclic chelates DOTA, NOTA and PCTA is shown in **Fig. 9**. Their synthesis in aqueous media with subsequent HPLC separation using reversed phase (C18) columns is quite simple and efficient with no need of additional purification. All starting components are commercially available.

The novelty of the synthesised conjugates lies in direct binding of [Lys^3]Bombesin to the core carbon of macrocyclic chelator which means that all carboxylic groups of the molecule are still available for complexation of radiometals. NOTA and especially PCTA should form stable radiometal complexes at much lower temperatures compared to commonly used DOTA [14-17].

However, some problems with stability of -NH-CS-NH- coupling bridge can appear as shown in **Fig. 6**. This bridge can be apparently splitted by hydrolysis in slightly alkaline solutions at pH > 8.

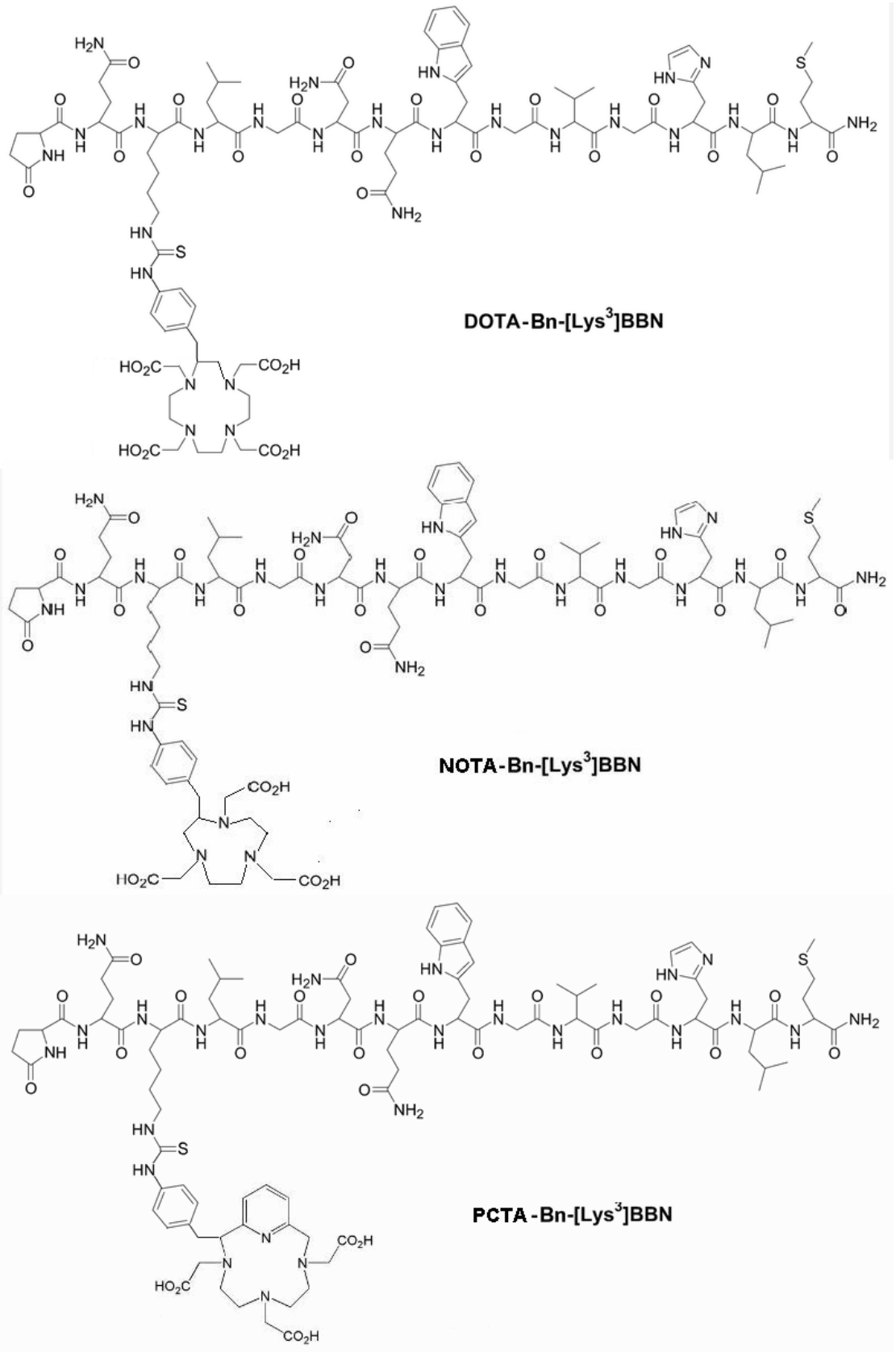


Figure 9: *Schematic structural formulas of the novel BFC - [Lys^3]Bombesin conjugates*

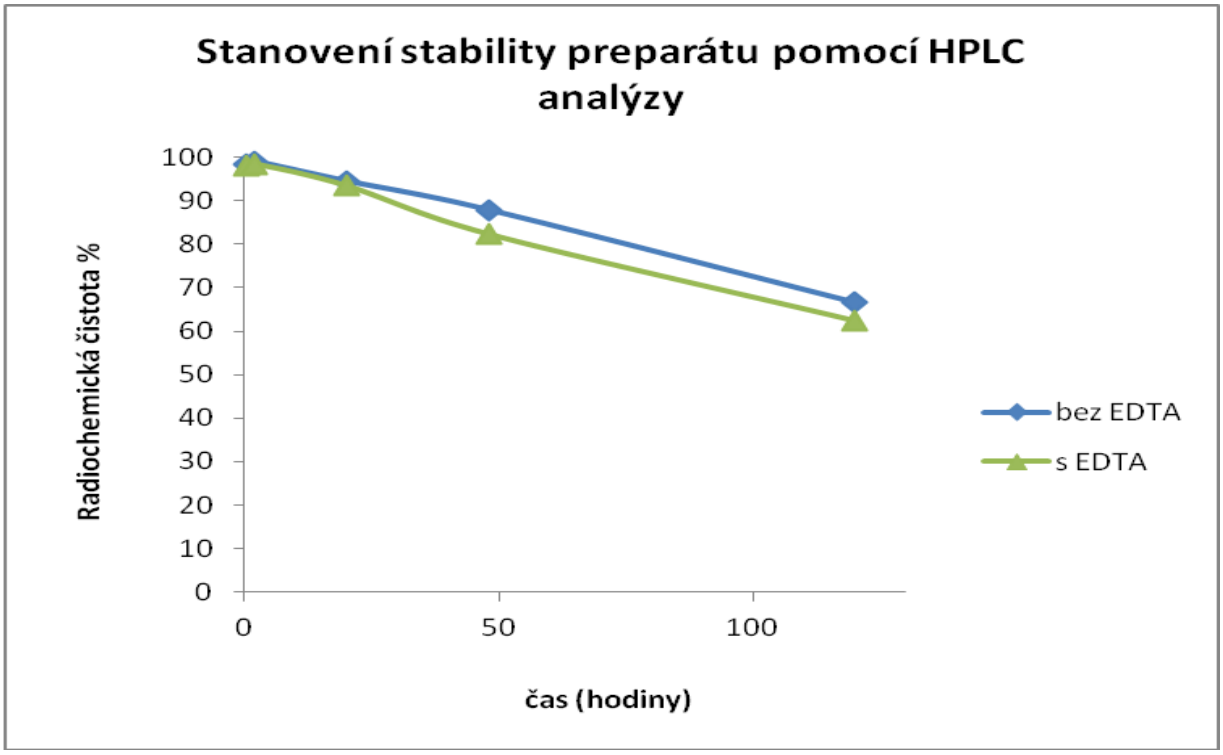


Figure 10: *Determination of the time dependence [hrs] of radiochemical purity of [^{177}Lu]PCTA[Lys^3]Bombesin at 4°C (green line - 5x10^{-4}M EDTA, blue line - without EDTA). Used with authors permission [18].*

First experiments with the newly synthesized radiolabeled conjugates were performed. Their basic behavior *in vitro* and *in vivo* was observed[18]. Labeling effeciency of PCTA[Lys^3]Bombesin with ^{177}Lu was > 98 % after 15 minutes at ambient laboratory temperature. The radiochemical purity during 5 days after labeling is shown in **Fig. 10**. Since nearly any released ^{177}Lu was complexed with EDTA, the decreasing radiochemical purity seems to be caused by splitting of the conjugate.

Further investigation of the prepared conjugates DOTA-p-Bz-NH-CS-NH-[Lys^3]Bombesin, NOTA-p-Bz-NH-CS-NH-[Lys^3]Bombesin and PCTA-p-Bz-NH-CS-NH-[Lys^3]Bombesin for radioimmunodiagnostics and therapy in oncology is required.

6. REFERENCES

[1] GLOBOCAN 2008, WHO - International Agency for Research in Cancer, Cancer Incidence, Mortality and Prevalence Worldwide in 2008, http://globocan.iarc.fr/factsheets/populations/factsheet.asp?uno=900.

[2] Anastasi, A., Erspamer, V., Bucci, M. (1971). Isolation and structure of bombesin and alytensin, two analogous active peptides from the skin of the European amphibians *Bombina* and *Alytes*, *Experimentia* **34**, 5-30.

[3] McDonald, T.J., Jörnvall, H., Nilsson, G., Vagne, M., Ghatei, M., Bloom, S.R., Mutt,V. (1978). Characterization of a gastrin releasing peptide from porcine non-antral gastric tissue, *Biochemical and Biophysical Research Communications* **90**, 227-233.

[4] Shuang Liu (2008). Bifunctional Coupling Agents for Radiolabeling of Biomolecules and Target-Specific Delivery of Metallic Radionuclides, *Adv Drug Deliv Rev* **60**, 1347–1370.

[5] Shuan Liu, Edwards, D.S. (2001). Bifunctional Chelators for Therapeutic Lanthanide Radiopharmaceuticals, *Bioconjugate Chem* **12**, 7-34.

[6] Seulki Lee**,** Jin Xie**,** Xiaoyuan Chen (2010). Peptide-based Probes for Targeted Molecular Imaging, *Biochemistry* **49**, 1364-1376.

[7] Kai Chen, Conti, P.S. (2010). Target-specific delivery of peptide-based probes for PET imaging, *Advanced Drug Delivery Reviews*, **60**, 1005-1022.

[8] Breeman, W.A.P., de Jong, M., Erion, J.L., Bugaj, J.E., Srinivasan, A., Bernard, B.F., Kwekkeboom, D.J., Visser, T.J., Krenning, E.P. (2002). Preclinical Comparison of ^{111}In-Labeled DTPA or DOTA-Bombesin Analogs for Receptor-Targeted Scintigraphy and Radionuclide Therapy, *J Nucl Med* **43**, 1650-1656.

[9] Biddlecombe, G.B., Rogers, B.E., de Visser, M., Parry, J.J., de Jong, M., Erion, J.L., Lewis, J.S. (2007). Molecular Imaging of Gastrin-Releasing Peptide Receptor-Positive Tumors in Mice Using ^{64}Cu- and ^{86}Y-DOTA-(Pro^{1},Tyr^{4})-Bombesin(1-14), *Bioconjugate Chem* **18**, 724-730.

[10] Cheng, K.T., Lewis, J.S., Biddlecombe, G.B. (2007). ^{64}Cu-DOTA-[Pro^{1},Tyr^{4}]-Bombesin[1-14], *Molecular Imaging and Contrast Agent Database (MICAD),* http://www.ncbi.nlm.nih.gov/books/NBK23640/ .

[11] Cheng, K.T., Lewis, J.S., Biddlecombe, G.B. (2007). ^{86}Y-DOTA-[Pro^{1},Tyr^{4}]-Bombesin[1-14], *Molecular Imaging and Contrast Agent Database (MICAD),* http://www.ncbi.nlm.nih.gov/books/NBK23575/ .

[12] Yi-Shan Yang, Xianzhong Zhang, Zhengming Xiong, Xiaoyuan Chen (2006). Comparative in vitro and in vivo evaluation of two ^{64}Cu-labeled bombesin analogs in a mouse model of human prostate adenocarcinoma, *Nucl Med Biol* **33**, 371-380.

[13] Xiaoyuan Chen, Park, R., Yingping Hou, Tohme, M., Shahinian, A.H., Bading, J.R., Conti, P.S. (2004). microPET and Autoradiographic Imaging of GRP Receptor Expression with ^{64}Cu-DOTA-[Lys^3]Bombesin in Human Prostate Adenocarcinoma Xenografts, *J Nucl Med* **45**, 1390-1397.

[14] Ferreira, C.L., Yapp, D.T., Lamsa, E., Gleave, M., Bensimon, C., Jurek, P., Kiefer, G.E. (2008). Evaluation of novel bifunctional chelates for the development of Cu-64-based radiopharmaceuticals, *Nucl Med Biol* **35**, 875-882.

[15] Ferreira, C.L., Yapp, D.T., Crisp, S., Sutherland, B.W., Ng, S.S.W., Gleave, M., Bensimon, C., Jurek, P., Kiefer, G.E. (2010). Comparison of bifunctional chelates for ^{64}Cu antibody imaging, *Eur J Nucl Med Mol Imaging*, **37**, 2117-2126.

[16] Ait-Mohand, S., Fournier, P., Dumulon-Perrault, V., Kiefer, G.E., Jurek, P., Ferreira, C.L., Bénard, F., Guérin, B. (2011). Evaluation of ^{64}Cu-labeled bifunctional chelate-bombesin conjugates, *Bioconjugate Chem* **22**, 1729-1735.

[17] Ferreira, C.L., Yapp, D.T., Raji, G., Jurek, P., Kiefer, G.E. (2011). Comparison of bifunctional chelates and isotopes for small peptide PET imaging, *J Label Compd Radiopharm* **54**, S1-S576.

[18] Lázníčková A. et. all. (2011). Bombesin analogs DOTA-[Lys3]Bombesin$_{1-14}$ and PCTA-[Lys3]Bombesin$_{1-14}$ labeled with ^{177}Lu, *Personal Communication* and *Internal Research Report No. 1/2011,* Pharmaceutical Faculty in Hradec Králové, Charles University in Prague.

7. LIST OF FIGURES: