SYNTHESIS OF MODIFIED PEPTIDES

Doctoral thesis

Orsolya Szolomájer-Csikós

Supervisor

Prof. Dr. Gábor K. Tóth

Chemistry Doctoral School

Department of Medicinal Chemistry

University of Szeged

Faculty of Sciences and Informatics

Szeged

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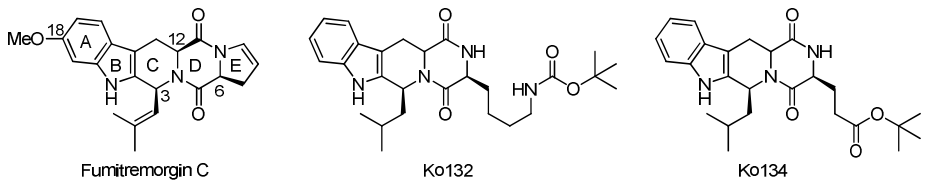
1. Introduction and aims

1.1 Design and synthesis of new fumitremorgin analogues

The human ABCG2 protein belongs to the ABCG subfamily of ABC transporters. The members of this subfamily are ABC half-transporters, they contain only one ABC and one transmembrane domain.

ABCG2 protein (placenta-specific ABC transporter/mitoxantrone resistance-associated protein/breast cancer resistance protein) has been identified recently as a candidate protein responsible for cancer multidrog resistance. The over expression of ABCG2 was found in several drug-selected cell lines and in tumorous tissues of patients. The activity of the human ABCG2 was suggested to be the major cellular defense mechanism against the cytotoxic drug, mitoxantrone, also several other drugs have been indicated as potential substrates of this drug pump.

Numerous ABCG2-specific inhibitors have been reported in the past decade. Such inhibitors include the antifungal agent fumitremorgin C (TFC), GF120918, Gleevec (imatinib mesylate), and so on (*Scheme 1.*). FTC, a member of a group of indole alkaloids, is a specific, selective inhibitor of the breast cancer resistance protein (BCRP/ABCG2).



Scheme 1. Some BCRP inhibitors

Our aims were to find a convenient strategy for optimizing Ko134 (published in literature) synthesis and to design new fumitremorgin analogues in order to increase specificity, chemical and metabolic stability of the original Ko134.

1.2 Synthesis of modified miniproteins

Understanding the mechanism behind protein folding, which is one of the most fundamental biochemical process, is proved to be a challenging task. Miniproteins represent simple and useful model systems in order to study the structural determinants of protein folding and stability. Miniproteins are adequate models to investigate various protein-

structure modifying effects such as temperature, pH, point mutation(s), H-bonds, salt bridges, molecular packing, etc.. TC5b, a 20-residue Trp-cage protein is one of the smallest of such models with a stable 3D fold, understanding the stability and folding of globular proteins.

Our aims were to design and synthesize TC5b miniprotein mutants, to better understand the global stability of the Trp-cage minipotein. Furthermore, Trp-cage miniprotein has been used to investigate the fold stabilizing effect of a salt bridge (Asp9-Arg16). Salt bridges are important as they contribute to protein folding, structure, residual flexibility and function. Salt bridges may increase the specificity of folding by reducing the number of way of molecular packing.

1.3 Synthesis of glycopeptides

Glycopeptides play a crucial role in various biological functions, especially in biological recognition events, signal transduction, and immune response. The carbohydrate moieties are attached through the oxygen in the side chain of serine/threonine in *O*-linked glycoproteins or through the carboxyamide nitrogen of asparagine in case of *N*-linked glycoproteins.

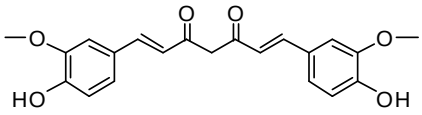
The rational preparation of the glycosylated peptides is still one of the most challenging tasks of peptide chemistry especially of those having oligosaccharide moieties. There are two main strategies: the stepwise approach (normally proceeds through a protected glycosylated amino acid intermediate which usually serves as the building block for a solid-phase construction of a peptide sequence) and the convergent method (the required carbohydrate chain and peptide are each built independently, and the amide or ester linkage is created in the synthesis). Both of them can be implemented in liquid or solid-phase.

Our aim was to design a mixed Fmoc/Boc synthesis strategy for the preparation of glycopeptides using a mild and selective deprotecting agent tin (IV)-chloride for the incorporation of Boc-protected glycosylated asparagine and serin derivatives.

1.4 Synthesis of precursors for radiopharmacon preparation

Alzheimer's disease is characterized by the presence of β-amyloid fibril formation. The inhibition of this peptide accumulation may be a prevention method for Alzheimer's disease. Beta-sheet breakers constitute a new class of drugs that are designed to specifically bind amyloid-beta peptide and block and/or reverse this abnormal conformational change. Several classes of molecules have been reported to inhibit β-amyloid fibril formation for example: Congo Red, some short fragments of the Aβ (RVVIA, LPFFD, LPYFD), curcumin (*Scheme 2*.).

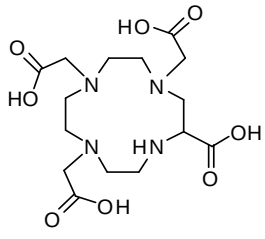
Curcumin (diferulomethane) is a low molecular weight molecule with potent antioxidant and anti-inflammatory activities that has a favorable toxicity profile and is under development as a potential cancer chemotherapeutic agent. The phenolic yellow curry pigment curcumin directly binds small β-amyloid species to block aggregation and fibril formation *in vitro* and *in vivo*. These data suggest that low dose curcumin effectively disaggregates Aβ as well as prevents fibril and oligomer formation, supporting the rationale for curcumin use in clinical trials preventing or treating Alzheimer's disease.



curcumin

Scheme 2.

Our aims were to design and synthesize precursors which can penetrate the blood brain barrier directly binds β-amyloid plaques and with the convenient Medical Image Processing which increases the chance of early detection of Alzheimer's disease. We wanted to design a combined solid and solution phase synthesis strategy for the incorporation of the curcumin, DOTA (*Scheme 3*.), *N*-carboxymethyl-histidine to the KLPYFD and LPYFD (short fragment of β-amyloid) peptides.



DOTA

Scheme 3.

2. Results and discussion

2.1 Design and synthesis of new fumitremorgin analogues

To improve the specificity and selectivity of Ko134 (fumitremorgin analogue) while eliminating the potential for toxicity, we have designed and synthesized a new class of FTC analogues using solid and solution phase synthesis strategy. To prepare larger quantities of Ko134 (published in literature) for *in vivo* studies, we have developed a straightforward and efficient solution phase synthesis strategy for the preparation of Ko134 inhibitor, using both RP-HPLC and column chromatography.

First we applied the solid phase synthesis strategy for the preparation of new fumitremorgin analogues. In solid phase, the elongation of the peptide chain with a Fmoc-protected amino acid to the tetrahydro-β-carboline's secondary nitrogen wasn't successful under various reaction conditions, even with the most potent coupling reagents: TCFH, TFFH, CIP, HATU. Therefore we decided to synthesize fumitremorgin analogues in solution phase. In solution phase incorporation of the Fmoc- and Boc-protected amino acids was successful using TCFH coupling reagent.

The FTC-Ko family proved to be a good starting point since these compounds displayed significant specificity that was confirmed in this study (Fig 25.). Our data show that the FTC type diketo-piperazine ring structure is essential for activity as tricyclic analogues IIIa-IIId showed no activity in the Hoechst assay. On the other hand, compounds that pereserved the diketo-piperazine ring structure were all active provided they had a *3S, 6S, 12aS* configuration (Fig 25.). Based on previous data, the compounds with *3S, 6R, 12aS* configuration were inactive as expected except perhaps the partially active 3e5 (*3S,6R,12aS*). But even in that case 3e5 (*3S,6S,12aS*) the diastereoisomeric pair was more than 110-fold more potent with IC50 values of 16.7 μM and 0.14 μM respectively. Remarkably, stereospecificity (*3S, 6S, 12aS vs 3S, 6R, 12aS)* observed in ABCG2 inhibition was completely absent in inhibition of ABCB1 and ABCC1 (Fig 25.). Moreover, the compounds with *3S, 6R, 12aS* configuration did not exhibit ABCG2 specificity over ABCB1 and ABCC1. The fact that configuration at position 6 alone confers specificity for ABCG2 over ABCB1 and ABCC1 has not been described before. This specificity confined to a single chiral center is quite unexpected considering the previosly described broad substrate specificity of these ABC transporters.

The **Ko134** *in vivo* biological analysis are in progress.

2.2 Synthesis of modified miniproteins

Hudaky et al. designed a new variant of TC6b, differs only by a methylene group from TC5b. Tc6b exhibits enhanced heat stability and adopts a stable fold at physiological temperature.

Based on this salt bridge optimized model system TC5b_D9E (NLYIQWLK**E**GGPSSG**R**PPPS) new miniprotein analogues were synthesized: TC5b_D9N (NLYIQWLK**N**GGPSSG**R**PPPS), TC5b_R16A (NLYIQWLK**D**GGPSSG**A**PPPS), TC5b_D9N_R16A (NLYIQWLK**N**GGPSSG**A**PPPS), TC5b_D9S (NLYIQWLK**S**GGPSSG**R** PPPS), TC5b_R16hR (NLYIQWLK**D**GGPSSG**hR**PPPS), TC5b_D9AaD_R16K (NLYIQWLK**AaD**GGPSSG**K**PPPS). The peptides were synthesized by solid-phase technique utilizing Fmoc-chemistry.

In order to monitor the structure stabilizing factor of the salt bridge Asp9-Arg16, the new TC5b mutants were studied at two different pHs (~3,0 and ~7,0), over a wide range of temperature relevant for proteins ($55 \leq T \leq 85°C$). Secondary structural changes recorded by ECD data were jointly analyzed and quantified by the deconvolution program CCA+. In order to locate structural differences at an atomic level in the fully folded states, high resolution H^1-H^1 NMR studies were completed (T= 280 K) both at neutral ($6,5 \leq pH \leq 3,2$) and acidic ($2,8 \leq pH \leq 3,2$) pH conditions. With the above model miniproteins and tools in hand, our aim was to evaluate the role of salt bridge on protein fold stability.

During the structural analysis, we found that the salt bridge of the Trp-cage scaffold is not an isolated structure stabilizing element but rather an integrated part of the foldamer. In case of the examined mutants the observed stability tendencies can be understand by considering three specific, but coupled interactions: electrostatic, helix-stabilizing (QxxxY) and hydrophobic [of the $–(CH_2)_3–$ arm of Arg16 with the indole ring of Trp6]. Based on the herein reported mutation studies it seems that the interaction network of the $–(CH_2)_3–$ arm of Arg16 residue is of higher importance, than the one operative between the negatively charged Asp9/Glu9 and the positively charged guanidine group. The elimination of one of the salt bridge forming partner (TC5b_D9S, TC5b_D9N) is less drastic than the elimination of the hydrophobic arm of Arg16 (TC5b_R16A).

The deconvolution of ECD spectra and the analysis of the acidic NMR spectra revealed that the melting scenario of the Trp-cage is not a simple two-state mechanism but rather a more comlex process with at least one intermedier state.

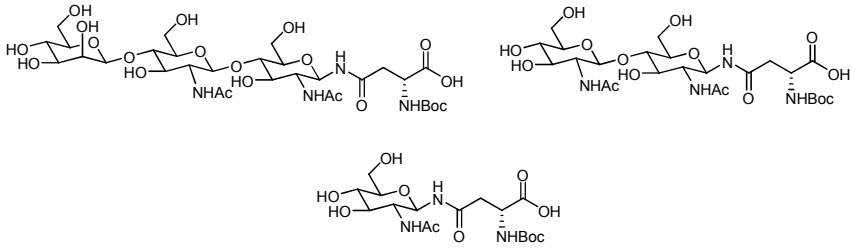
Protein aggregation and misfolding of proteins can be linked to the origin of many conformational diseases which can be either genetic or spontaneous. The proteins involved can either have an unstructured or lineal unfolded form such as in Alzheimer's and Parkinson's disease or Type II Diabetes, or can be globular, showing a folded 3D-structure. Protein aggregation is a major importance in biomedicine, yet it is not well understand. In the framework of our research project on the structural analysis of TC5b analogues we observed aggregation in case of TC5b_D9N and other TC5b phosphorylated mutants. Consequently, TC5b structure can serve as a good model for Alzheimer's aggregates. Based on our previous studies new TC5b analogues were synthesized for further structural investigations: TC5b_D9Q (NLYIQWLK**Q**GGPSSG**R**PPPS), TC5b_S14E (NLYIQWLK**D**GGPS**E** G**R**PPPS), TC5b_S14Q (NLYIQWLK**N**GGPS**Q**G**R**PPPS), TC5b_S20E(NLYIQWLK**N**G GPSSG**R**PPP**E**), TC5b_S20Q (NLYIQWLK**N**GGPSSG**R**PPP**Q**).

2.3 Synthesis of glycopeptides

For the preparation of glycopeptides a mixed Fmoc/Boc solid-phase synthesis strategy was applied by using a new, mild and selective Boc deprotecting agent.

According to literature the Fmoc-protected glycosylated asparagine and serine derivatives proved to be suitable building blocks for the solid-phase peptide synthesis. In some cases the preparation of Boc-protected glycosylated amino acid building blocks are more convenient than the Fmoc protected ones. For the incorporation of the Boc-protected glycosylated amino acid derivatives in the model peptides selective Boc deprotecting agent tin(IV) chloride was used. The glycopeptides were synthesized using Fmoc chemistry on a TFA cleavable Rink-amide MBHA resin. According to our investigations other resins commonly used in Fmoc chemistry (Rink amide, 2-chlorotrityl resin, Wang resin) showed leakage of the peptide from the resin during cleavage with tin(IV) chloride, except the Rink-amide MBHA.

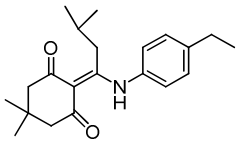
As model peptides we used a shorter fragment of the Trp-cage miniprotein (Leu-Lys-Asn*-Gly-Gly-Pro) and an aggrecan fragment, from the most glycosylated region of the protein (Gly-Val-Glu-Asp-Ile-Ser*-Gly-Leu-Pro-Ser-Gly). (Where * is site of glycosylation).



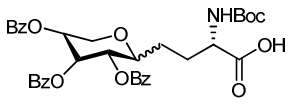
Scheme 4.

For the synthesis of Leu-Lys-[GlcNAc(β1-N)]Asn-Gly-Gly-Pro-NH_2,Leu-Lys-[GlcNAc(β1-4)GlcNAc (β1-N)]Asn-Gly-Gly-Pro-NH_2, Leu-Lys-[Man(β14)GlcNAc(β1-4)GlcNAc(β1-N)]Asn-Gly-Gly-Pro-NH_2 Rink amide-MBHA resin was applied. As building blocks N,α-Boc-protected glycosylated asparagine derivatives were used (*Scheme 4.*). The first 3 amino acids were incorporated using Fmoc strategy. After coupling of Boc-protected glycosylated aspartic acid derivatives, the resin was treated twice with 0.2 M $SnCl_4$, giving the resin a red colour due to complex formation with $SnCl_4$. Coupling of the last two amino acid was performed using Fmoc chemistry.

The synthesis strategy of H-Gly-Val-Glu-Asp-Ile-[Xil(β1-O)]Ser-Gly-Leu-Pro-Ser(Bzl)-Gly-NH_2 was the same as in case of *N*-glycopeptides in exception that in this case the peptide contains a glycosylated serin derivative with three unprotected hydroxyl functional groups and several trifunctional amino acids, which side chains must be protected during the synthesis. Based on our earlier research the Ser-Bzl, Ser(Xil)-Bz, Glu-, Asp-ODmab (*Scheme 5*.) protecting group combination was suitable for the preparation of this glycopeptide. As building block N,α-Boc-protected serin derivative was used (*Scheme 6.*). Dmab-deprotection was made with a solution of hydrazine-hydrate, on resin. Bz-deprotection was made in solution-phase with hydrazine-hydrate.



Scheme 5. Dmab protecting group



Scheme 6. Boc-Ser[Xil(OBz)$_3$]-OH

In summary we found a convenient solid-phase synthesis strategy for the preparation of the above glycosylated-hexapeptide conjugates. I have developed a strategy that combines the Fmoc and Boc SPPS approaches for the preparation of *N*- and *O*-glycopeptides in high purity and reasonable yield, using $SnCl_4$ for Boc deprotection, which leaves the acid sensitive glycosidic bonds intact.

2.3 Synthesis of precursors for radiopharmacon preparation

For the preparation of the precursors, amyloid peptide mimics LPYFD, KLPYFD, curcumin, DOTA and *N*-carboxymethyl-histidine were chosen.

In the framework of our project on the synthesis of precursors in solution phase, phenolic hidroxyl group of the curcumin was alkylated with ethyl bromoacetate. The resulting curcumin derivative was then used to create a curcumin-peptide, but incorporation wasn't successful.

Therefore we decided to modify the strategy for the incorporation of curcumin in the model peptide by using a new mixed solution and solid phase synthesis strategy. First the short fragments of the β-amyloid peptide LPYFD and KLPYFD were synthesized manually using Fmoc peptide synthesis protocols. Henceforth, bromo-acetyl peptide was used for coupling with curcumin (in solid phase) then curcumin-peptide was acylated with DOTA in solution phase. For the incorporation of *N*-carmoxymethyl histidine the same synthesis strategy was used, as well the synthesized bromo-acetyl peptide was used for coupling with *N*-carmoxymethyl histidine.

In summary, we found a convenient strategy for the preparation of the above precursors, using a combined solid and solution phase synthesis. With this combined technique incorporation of the curcumin, DOTA and *N*-carboxymethyl-histidine was successful. Analysis concerning the new biological activity are in progress.

Publications related to this doctoral thesis

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2. Petra Rovó, Viktor Farkas, Orsolya Hegyi, **Orsolya Szolomajer-Csikós**, Gábor K. Tóth, András Perczel: Cooperativity network of Trp-cage miniproteins: probing salt-bridges, *Journal of Peptide Science (2011),* **17**(9), (IF = 1,954)

3. Kinga Rákosi, **Orsolya Szolomajer-Csikós**, László Kalmár, Zoltán Szurmai, János Kerékgyártó, Gábor K. Tóth: Synthesis of N-glycopeptides applying glycoamino acid building blocks with a combined Fmoc/Boc strategy, *Protein & Peptide Letters* (2011), **18**(7), (IF = 1,755).

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3. Rákosi Kinga, **Szolomájer-Csikós Orsolya**, Hegyi Orsolya, Váradi Györgyi, Kalmár László, Kerékgyártó János, Tóth Gábor K.: Glikopeptidek szintézise - egy szintetikus kihívás, Cluj-Napoca, Romania, Nov. 13-15, 2008.

4. Rákosi Kinga, **Szolomájer-Csikós Orsolya**, Hegyi Orsolya, Kovács Anita, Váradi Györgyi, Kalmár László, Kerékgyártó János, Tóth Gábor K.: *O*-glikopeptidek szintézisének lehetőségei, XV. Nemzetközi Vegyészkonferencia, Targu-Mures, Romania, Nov. 12-15, 2009.