

A novel approach in potential anticoagulants from peptides epitope 558–565 of A2 subunit of factor VIII

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Abstract Factor VIII, a human blood plasma protein, plays an important role during the intrinsic pathway of blood coagulation cascade after its activation by thrombin. The activated form of FVIII acts as cofactor to the serine protease Factor IXa, in the conversion of the zymogen Factor X to the active enzyme Factor Xa. The Ser⁵⁵⁸–Gln⁵⁶⁵ region of the A2 subunit of Factor VIII has been shown to be crucial for FVIIIa–FIXa interaction. Based on this, a series of linear peptides, analogs of the 558–565 loop of the A2 subunit of the heavy chain of Factor VIII were synthesized using the acid labile 2-chlorotriyl chloride resin and biologically evaluated in vitro by measuring the chronic delay of activated partial thromboplastin time and the inhibition of Factor VIII activity, as potential anticoagulants.

Keywords Factor VIII · Anticoagulant activity · Activated partial thromboplastin time · Synthetic peptides · Blood coagulation cascade · FVIII-FIX interaction

Abbreviations

AcOH	Acetic acid
aPTT	Activated partial thromboplastin time
Boc	Tert-butoxycarbonyl
Bzl	Benzyl group
CLTR-Cl	2-chlorotriyl chloride
DCM	Dichloromethane
DIC	N,N'-diisopropylcarbodiimide

DIPEA	Diisopropylethylamine
DMF	N,N'-dimethylformamide
dPPP	Deficient platelet poor plasma
ESI-MS	Electrospray ionization mass spectrometry
Fmoc	9-Fluorenylmethyloxycarbonyl group
FVIII	Factor VIII
FIX	Factor IX
HOBt	1-hydroxybenzotriazole
i-PrOH	2-propanol
Me	Methyl group
MeCN	Acetonitrile
MeOH	Methanol
MBHA	4-methylbenzhydrylamine
PPP	Platelet poor plasma
PyBOP	(Benzotriazol-1-yloxy)-tris(pyrrolidino)phosphonium hexafluorophosphate
PT	Prothrombin time
rFVIII	Recombinant Factor VIII
tBu	Tert-butyl group
RP-HPLC	Reversed phase high performance liquid chromatography
TES	Triethylsilane
TFA	Trifluoroacetic acid
TFE	2,2,2-trifluoroethanol
TLC	Thin layer chromatography
Tol	Toluene
Trt	Trityl group

Abbreviations of common amino acids are in accordance with the recommendations of IUPAC-IUB Joint Commission on Biochemical Nomenclature: Arch Biochem Biophys 206 (1988) v–xxii, J Biol Chem 264 (1989) 668–673, J Peptide Sci 12 (2006) 1–8.

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Introduction

Cardiovascular diseases (CVDs) are the most common cause of morbidity and mortality worldwide (Mahan and

Fanikos 2011). Hemostasis, from Greeks meaning blood-stop, is the essential human mechanism to prevent blood loss in case of blood vessel injury, whereas the pathological clot formation, that is thrombosis, is the mechanism when hemostasis is excessively activated in the absence of bleeding. In these abnormal conditions, clot formation is prevented using anticoagulant drugs. Current anticoagulant therapies (Vitamin K antagonists, heparins, direct inhibitors of Factor Xa, etc.) have their limitations, so identification of new targets for blocking thrombosis without increasing the risk of severe and fatal bleeding is the major goal in anticoagulation therapy. These include inhibitors of the factors VIIa, Xa, IXa and VIIIa (Minors 2007; Lin et al. 2006; Howard et al. 2007; Franchini and Mannucci 2011).

Clotting is the result of a combined cascade of two biochemical pathways: “intrinsic pathway”, so called because all components are present in blood, and “extrinsic pathway”, in which tissue factor (TF) is required in addition to circulating components. Although during the previous decades the intrinsic pathway was postulated to play a less significant role; recently, its important participation has been shown in sustaining thrombus development in vivo (Gailani and Renne 2007; Woodruff et al. 2010). FVIIIa forms a heterotrimer complex with FIXa, Ca^{2+} (which come from activated platelets) and negatively charged phospholipids of the cells membrane. This complex, called tenase, converts Factor X to the activated form FXa, another protease that binds to cofactor Va to generate a complex known as prothrombinase. The latter complex converts prothrombin to thrombin which acts on fibrinogen to generate fibrin monomer, which is polymerized rapidly to form fibrin clot.

Factor VIII is composed of a large number of amino acid residues arranged within six domains known as $\text{A}_1\text{--A}_2\text{--B--A}_3\text{--C}_1\text{--C}_2$ (Vehar et al. 1984). The FVIII protein is secreted as a heterodimer including a heavy chain ($\text{A}_1\text{--A}_2\text{--B}$) and a light chain ($\text{A}_3\text{--C}_1\text{--C}_2$). Both chains are highly associated with a lipoprotein receptor related protein (LRP). FVIII that circulates in blood as a complex multimeric protein with von Willebrand factor is synthesized by endothelial cells (Fay 2004). This protein, vWF, is present in plasma and has been released in the subendothelial tissue of human saphenous and umbilical veins (Terraube et al. 2010). Its main role is the engagement in other proteins, particularly in factor VIII and consequently it is important in the adherence of platelets at the points of wounded vessels (Terraube et al. 2010). Factor VIII is actually inactive as a cofactor in blood coagulation, but is converted into its active cofactor form by proteolytic cleavage (Fang et al. 2007). The A_2 subunit includes an epitope, residues 558–565, which interacts with FIXa, residues 330–338, and it is likely the major catalytic interaction between the

FVIIIa and FIXa (Fay et al. 1994; O'Brien et al. 1995; Bajaj et al. 2001; Shen et al. 2008; Ngo et al. 2008).

Synthetic peptides or peptidomimetics which show that block the interaction–formation of the FVIIIa–FIXa complex could be used as leading compounds for novel anti-coagulant drugs. Here, we present the synthesis and biological evaluation of linear peptides, analogs of the sequence 558–565 of the A_2 subunit, aiming at the inhibition of FVIIIa–FIXa interaction, and studying their anticoagulant activity in vitro.

Reagents and methods

The peptides were prepared by solid-phase peptide synthesis techniques. The 9-fluorenylmethoxycarbonyl (Fmoc) protected amino acids and coupling reagents (HOBt, DIC, PyBOP, etc.) as well as 2-chlorotrityl chloride resin (CLTR-Cl) and MBHA Rink amide linker, used as solid support, were purchased from CBL-Patras (Patras, Greece). All the solvents and reagents used for solid-phase synthesis were of analytical grade and were used without further purification. Dichloromethane (DCM) was distilled over CaH_2 before usage. Thin layer chromatography (TLC) was carried out on precoated silica gel F-254 plates (Merck, Darmstadt, Germany). HPLC-grade solvents were also purchased from Merck (Darmstadt, Germany) whereas ESI–MS spectra were recorded on a Waters Micromass ZQ 4000 mass detector (positive mode), controlled by Mass-Lynx 4.1 software.

Synthesis

Anchoring on the resin

The examined peptides were synthesized using CLTR-Cl or MBHA Rink amide linker resins by Fmoc solid-phase peptide synthesis protocol. The esterification of the first Fmoc-amino acid onto the CLTR-Cl was carried out as first described by Barlos et al. (1991). CLTR-Cl (1 g) was inflated in DCM (10 mL) for 10 min. Fmoc-amino acid (1 mmol) was then added and the mixture was stirred gently in the presence of DIPEA (2.5 mmol) for 45 min at RT. The remaining active sites of the resin were capped using a mixture of MeOH/DIPEA/DCM (10:5:85) ($3 \times 5 \text{ mL} \times 10 \text{ min}$) at RT. The Fmoc-amino acid-resin was filtered and washed successively with DCM ($3 \times 5 \text{ mL}$), DMF ($3 \times 5 \text{ mL}$), 2-propanol ($3 \times 5 \text{ mL}$) and *n*-hexane ($1 \times 5 \text{ mL}$) and dried over P_2O_5 under vacuum. Subsequently, the Fmoc group was removed by treatment with a solution of 20 % piperidine in DMF for 30 min.

Coupling procedure

For the coupling reactions the method of carbodiimides was applied. In particular, Fmoc-amino acid (3 equiv of the resin substitution) and HOBt (4.5 equiv of the resin substitution) were dissolved in DMF (1–3 mL) and DIC (3.3 equiv of the resin substitution) was added. The progress and the completeness of each coupling was verified by the Kaiser's test and TLC, using MeCN/H₂O (5:1), CHCl₃/MeOH/AcOH (85:10:5) or Tol/MeOH/AcOH (7:1.5:1.5) as development solvents. (The TLC procedure is as follows: a few beads of resin were placed in a very small flask and 2–5 drops of TFA cocktail were added. After a short mixing, the mixture was left at room temperature for 15–20 min. TLC was then applied and the plate was visualized under UV lamp at 254 nm followed by ninhydrin spraying. In case of uncompleted reaction a magenta spot was near the start line of TLC.) The Fmoc group deprotection was performed as described above. All coupling and deprotection steps have been repeated until the expected sequence to be complete.

Cleavage from the resin

The synthesized protected peptide was cleaved from the resin in two steps: firstly, the peptide–resin ester was cleaved with a mixture of DCM/TFE (70:30) for 1 h at RT. The resin was filtered off and the solvent was removed on a rotary evaporator. The obtained crude protected peptide was washed with DCM and precipitated by adding cold and dried diethyl ether as a white solid. In a second step, the side chain deprotection of the linear protected peptide was achieved using a mixture of TFA/DCM/TES (90:5:5) and stirred gently for 3 h at RT. The solvent was evaporated under vacuum; the crude free peptide was precipitated with cold dried diethyl ether and collected by filtration (Fig. 1).

For the peptides attached onto the MBHA Rink amide resin the coupling and deprotection steps were performed as mentioned above. After the completeness of the desired sequence, the cleavage of the peptide from the resin as well as the deprotection of the side protected groups were performed in one step using a mixture of TFA/DCM/TES (90:5:5) by stirring gently for 3 h at RT. The solvent was evaporated under vacuum and the crude free peptide was precipitated with cold and dried diethyl ether and collected by filtration.

Purification and characterization of the crude peptides

The crude intermediates as well as the final compounds were purified on a semi-preparative RP-HPLC (Marathon IV pumps combined with a Fasma 500 UV detector, Rigas Labs, Greece) using a RP-HPLC Nucleosil C-18 column

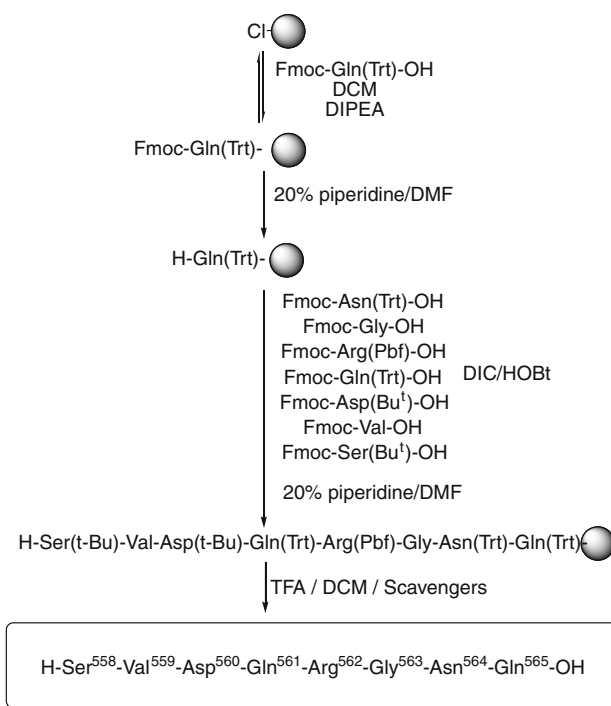


Fig. 1 Synthetic procedure of linear analogs of the Sequence Ser⁵⁵⁸–Gln⁵⁶⁵

(250 mm × 10 mm, 7 μm) by gradient elution of 5–85 % solvent B (solvent A: 0.1 % TFA in H₂O; solvent B: 0.1 % TFA in MeCN) over 70 min at a flow rate of 2.5 mL/min at 214 and 254 nm. All analytical chromatograms and mass spectra (ESI–MS) were recorded on a Waters Alliance 2695LC HPLC system with a Waters 2966 Photodiode Array detector coupled to a Waters Micromass ZQ mass spectrometer using a RP-Nucleosil C-18 column (250 × 4.6 mm, 5 μm) at 214 and 254 nm. Separation was achieved by gradient elution under the following conditions: S1, 0–30 % solvent B over 30 min at a flow rate of 1 mL/min; S2, 0–40 % solvent B over 30 min at a flow rate of 1 mL/min (solvent A: 0.1 % TFA in water; solvent B: 0.1 % TFA in MeCN).

Biological: anticoagulant assays

Measurements were performed on an automatic analyzer ACL Elite Pro whereas hemostasis reagents were purchased from the Instrumentation Laboratory, UK and recombinant FVIII (rFVIII) was purchased from Baxter AG, USA. The assays were performed in triplicate and the results were expressed as the average of measurements for each sample. Blood from healthy individuals was collected into plastic tubes containing 3.8 % trisodium citrate as anticoagulant (blood/trisodium citrate, 9:1). Citrated blood was immediately centrifuged at 13,000 rpm for 10 min at

room temperature to obtain platelet poor plasma (PPP). Citrated samples can be run within 2 hours of sample collection.

Measurement of activated partial thromboplastin time (aPTT) assay

An aliquot of PPP (200 μ L) was transferred into a vial and peptide solution (200 μ L, 1 mg/mL in Owren–Koller buffer, pH 7.4) was added and the mixture was incubated at 37 °C for 30 min. The control solution was comprised of PPP (200 μ L) and buffer (200 μ L) and was incubated under the same conditions. After that, the incubated peptide solution (10 μ L) was transferred to the instrument cuvette as well as cephalin–kaolin solution (10 μ L, 20 mg/mL kaolin and a 1:10 dilution of bovine brain cephalin in 154 mmol/L NaCl), acting as aPTT contact activator (Brunnee et al. 1993). The cuvettes were transferred to the measuring position and CaCl_2 (10 μ L, 25 mM) was added to prolong further reaction. Time required for clot formation was then reported and the divergent time was extracted by the instrument. Phospholipids as well as Ca^{2+} are essential for activation of FX and fibrin clot formation.

Prothrombin time (PT) assay tests the overall efficiency of the extrinsic pathway. PT reagent (tissue factor and phospholipids) and calcium ions (CaCl_2) are added to pre-incubated plasma and clotting time is recorded directly.

Inhibition of FVIII activity assay

If Factor VIII is added to plasma containing a FVIII antagonist and the mixture is incubated, then the FVIII is progressively inactivated. This assay can be performed using human or porcine recombinant FVIII. The presence of the antagonist is associated with the reduced activity of FVIII, which was determined using aPTT assay with FVIII deficient platelet poor plasma (dPPP) instead of normal one.

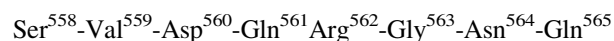
An aliquot of rFVIIIa (200 μ L, diluted (1:1) in Owren–Koller buffer pH 7.4, 1 U/mL) was transferred into a vial containing peptide solution (200 μ L, 1 mg/mL in Owren–Koller buffer, pH 7.4) and incubated at 37 °C for 30 min. Control 1 was comprised of rFVIIIa (400 μ L, diluted (1:1) in Owren–Koller buffer pH 7.4, 1 U/mL) whereas Control 2 contained rFVIIIa (200 μ L diluted (1:1) in Owren–Koller buffer pH 7.4, 1 U/mL) and buffer (200 μ L). All the sample vials were incubated at 37 °C for 30 min. Then, the incubated peptide solution (10 μ L) was transferred to the instrument cuvette as well as cephalin–kaolin solution (10 μ L), acting as aPTT contact activator and dPPP (deficient PPP to FVIII, 10 μ L). The cuvette was transferred to the measuring position and CaCl_2 (25 mM, 10 μ L) was added to prolong further reactions. Inhibition values were

reported and extracted automatically by the instrument (Barrowcliffe et al. 2002).

Results and discussion

Anticoagulants inhibit thrombin and fibrin formation and are used widely for the treatment of venous thromboembolism. The main anticoagulants used by physicians over 60 years are heparin and warfarin. However, the limitations of these medicines have led the pharmaceutical industry to search for new targets and develop more efficient compounds without the side-effects of current anticoagulants (like bleeding complications, interactions with other drugs, dietary implications, etc.) (Gailani and Renne 2007). Current knowledge highlights the importance of FVIIIa–FIXa interaction in clot formation. Thus, the interaction sites represent excellent targets for the anticoagulant therapy (Lenting et al. 2010; Mahan and Fanikos 2011).

Our research effort is focused on the synthesis of peptides (Sarigiannis et al. 2006), analogs of the sequence Ser⁵⁵⁸–Gln⁵⁶⁵ of the A2 subunit of FVIIIa that interacts with FIXa, aiming to block the FVIIIa–FIXa complex formation, to suspend platelets adhesion and furthermore clot formation.



Here, we present a series of synthetic linear peptides incorporating Gln, Glu, Asp and their side chain benzyl and methyl esters instead of Asn⁵⁶⁴ as well as the N^α-acetyl peptidic analogs and their biological effect acting as anti-coagulant compounds. They are based on point mutations within the 558–565 region resulting in severe or mild hemophilia A, such as Asp⁵⁶⁰–Ala, mild; Val⁵⁵⁹–Ala, mild; Ser⁵⁵⁸–Phe, mild; etc. (Fay and Jenkins 2005; Jenkins et al. 2002).

All the analogs shown in Table 1 were synthesized manually using as solid support the 2-chlorotriyl chloride resin or Rink amide MBHA resin and standard coupling procedures for Fmoc/Bu^t methodology. The nature of the resins used for peptide synthesis as well as the efficient protection of the side chain of Gln residues with Trt group yielded final products with high purity. Nevertheless, these crude products were purified by semi-preparative HPLC as reported above. The appropriate fractions containing the desired products were lyophilized to give a white fluffy solid. The final verification of the peptide sequence was achieved by ESI-MS (Micromass, UK). An example of analytical RP-HPLC chromatogram of purified peptide and ESI-MS spectrum is shown in Figs. 2 and 3, respectively for the analog 7.

The synthesized analogs were investigated for their anticoagulant activity by measuring their activated partial

Table 1 Analytical data of the synthesized peptides

Synthesized analogs	$M_{\text{calculated}}$	M_{found}	t_R (min)
1. Ser ⁵⁵⁸ -Val ⁵⁵⁹ -Asp ⁵⁶⁰ -Gln ⁵⁶¹ -Arg ⁵⁶² -Gly ⁵⁶³ -Asn ⁵⁶⁴ -Gln ⁵⁶⁵ -OH	902.9	903.5	5.92 ^a
2. Ser ⁵⁵⁸ -Val ⁵⁵⁹ -Asp ⁵⁶⁰ -Gln ⁵⁶¹ -Arg ⁵⁶² -Gly ⁵⁶³ -Asp ⁵⁶⁴ -Gln ⁵⁶⁵ -OH	903.4	904.1	7.67 ^a
3. Ser ⁵⁵⁸ -Val ⁵⁵⁹ -Asp ⁵⁶⁰ -Gln ⁵⁶¹ -Arg ⁵⁶² -Gly ⁵⁶³ -Asp(OMe) ⁵⁶⁴ -Gln ⁵⁶⁵ -OH	917.9	918.5	8.95 ^a
4. Ser ⁵⁵⁸ -Val ⁵⁵⁹ -Asp ⁵⁶⁰ -Gln ⁵⁶¹ -Arg ⁵⁶² -Gly ⁵⁶³ -Asp(OBzl) ⁵⁶⁴ -Gln ⁵⁶⁵ -OH	994.0	994.7	12.77 ^a
5. Ser ⁵⁵⁸ -Val ⁵⁵⁹ -Asp ⁵⁶⁰ -Gln ⁵⁶¹ -Arg ⁵⁶² -Gly ⁵⁶³ -Gln ⁵⁶⁴ -Gln ⁵⁶⁵ -OH	916.9	917.4	5.42 ^a
6. Ser ⁵⁵⁸ -Val ⁵⁵⁹ -Asp ⁵⁶⁰ -Gln ⁵⁶¹ -Arg ⁵⁶² -Gly ⁵⁶³ -Glu ⁵⁶⁴ -Gln ⁵⁶⁵ -OH	917.9	918.5	9.35 ^b
7. Ser ⁵⁵⁸ -Val ⁵⁵⁹ -Asp ⁵⁶⁰ -Gln ⁵⁶¹ -Arg ⁵⁶² -Gly ⁵⁶³ -Asn ⁵⁶⁴ -Gln ⁵⁶⁵ -NH ₂	901.9	902.6	8.22 ^b
8. Ac-Ser ⁵⁵⁸ -Val ⁵⁵⁹ -Asp ⁵⁶⁰ -Gln ⁵⁶¹ -Arg ⁵⁶² -Gly ⁵⁶³ -Asn ⁵⁶⁴ -Gln ⁵⁶⁵ -OH	945.0	945.6	11.58 ^a
9. Ac-Ser ⁵⁵⁸ -Val ⁵⁵⁹ -Asp ⁵⁶⁰ -Gln ⁵⁶¹ -Arg ⁵⁶² -Gly ⁵⁶³ -Asp ⁵⁶⁴ -Gln ⁵⁶⁵ -OH	945.9	946.5	13.15 ^a
10. Ac-Ser ⁵⁵⁸ -Val ⁵⁵⁹ -Asp ⁵⁶⁰ -Gln ⁵⁶¹ -Arg ⁵⁶² -Gly ⁵⁶³ -Asp(OMe) ⁵⁶⁴ -Gln ⁵⁶⁵ -OH	960.0	960.6	15.82 ^a
11. Ac-Ser ⁵⁵⁸ -Val ⁵⁵⁹ -Asp ⁵⁶⁰ -Gln ⁵⁶¹ -Arg ⁵⁶² -Gly ⁵⁶³ -Asp(OBzl) ⁵⁶⁴ -Gln ⁵⁶⁵ -OH	1036.1	1036.8	19.20 ^a
12. Ac-Ser ⁵⁵⁸ -Val ⁵⁵⁹ -Asp ⁵⁶⁰ -Gln ⁵⁶¹ -Arg ⁵⁶² -Gly ⁵⁶³ -Gln ⁵⁶⁴ -Gln ⁵⁶⁵ -OH	959.0	959.8	16.57 ^a
13. Ac-Ser ⁵⁵⁸ -Val ⁵⁵⁹ -Asp ⁵⁶⁰ -Gln ⁵⁶¹ -Arg ⁵⁶² -Gly ⁵⁶³ -Glu ⁵⁶⁴ -Gln ⁵⁶⁵ -OH	960.0	960.7	16.99 ^b
14. Ac-Ser ⁵⁵⁸ -Val ⁵⁵⁹ -Asp ⁵⁶⁰ -Gln ⁵⁶¹ -Arg ⁵⁶² -Gly ⁵⁶³ -Asn ⁵⁶⁴ -Gln ⁵⁶⁵ -NH ₂	944.0	944.6	12.42 ^b

^a Linear gradient 0–30 % solvent B for 30 min

^b Linear gradient from 0–40 % solvent B for 30 min, (solvent A: 0.1 % TFA in water; solvent B: 0.1 % TFA in MeCN)

thromboplastin time (aPTT) and prothrombin time (PT) assay, a sensitive screen method for the evaluation of the intrinsic, extrinsic and common coagulation pathways. PT and aPTT tests are the suggested protocols when a coagulation disorder is suspected, for monitoring anticoagulant therapy, as well as for a preoperative screening. Light transmission is used to determine PT and aPTT times. A change in the light transmittance due to clot formation is converted through a microcomputer as determined coagulation time.

Prolonged clotting time is observed in case of factor deficiencies, liver disease, vitamin K deficiency, heparin presence or other inhibitors, whereas a shortened aPTT is associated with the risk of venous thromboembolism. A long aPTT with normal PT indicates inhibition of coagulation process or bleeding disorders due to deficiencies of factors VIII, IX, XI and XII or the presence of a coagulation inhibitor. Abnormalities in the levels of factors VIII, IX, X, XI or XII have an impact on aPTT, which measures only the integrity of the intrinsic pathway. Short aPTT has been associated with a significantly increased risk of thrombotic events (CLSI 2008). A prolongation of aPTT over 4 s ($\text{aPTT}_{\text{sample}} - \text{aPTT}_{\text{control}} > 4$ s) is remarkable.

The Fig. 4 summarizes the effect of the synthesized peptides on the aPTT assay. It is obvious that analogs 1, 2 and 7 are the most active. Analog 1 shows a prolonged aPTT of 5.8 s whereas analog 2, that incorporates Asp⁵⁶⁴ instead of Asn, displays similar activity (aPTT: 5.0 s). Furthermore, a quite analogous aPTT (5.1 s) is observed for analog 7. The results show that the native sequence incorporating either C-terminal carboxy or amide group retards clotting formation; more active is the C-terminal

carboxy group by comparing analogs 1 and 7. On the opposite, analogs 3 and 4 result in a significant loss of activity in aPTT assay, likely due to the incorporation of aspartic acid β -methyl or β -benzyl ester, respectively. Especially, analog 3 accelerates aPTT more than all the other synthesized peptides, two to fourfold drastic loss of activity. Replacement of Asn⁵⁶⁴ with Gln (5) or Glu (6), e.g. elongation of the side chain of amino acid at the position 564, doesn't have any significant result, thus Asn⁵⁶⁴ appears to be crucial for the prolongation of aPTT. In addition, all the N^α-acetyl analogs reduce aPTT, something rather not usually observed. Furthermore, all the tested peptides show normal PT as it was expected; in case of abnormal PT interaction of the peptide with the factors of extrinsic pathway have to be tested. This, associated with the above results strengthens further the evidence of the role of the peptides 1, 2 and 7 only in the intrinsic coagulation pathway to be evolved.

Another useful and more specific test for monitoring hyper-coagulable state is Factor VIII activity. We have examined the influence of these synthetic peptides on the interaction between FVIIIa and FIXa as a reduction of the FVIIIa activity using rFVIIIa in human plasma deficient to FVIII. In order to confirm the presence of the other coagulation factors at normal levels, PT assay applied first. rFVIII is added to deficient platelet poor plasma (dPPP) containing a synthetic peptide and the mixture is incubated. A reduced FVIIIa activity is observed when a synthetic peptide acts as inhibitor.

The Fig. 5 represents the % inhibition of the FVIIIa activity due to the synthesized peptides. Analogs 1, 6 and 12 cause the highest % inhibition of FVIIIa activity.

Fig. 2 RP-HPLC of the purified analog Ser-Val-Asp-Gln-Arg-Gly-Asn-Gln-NH₂

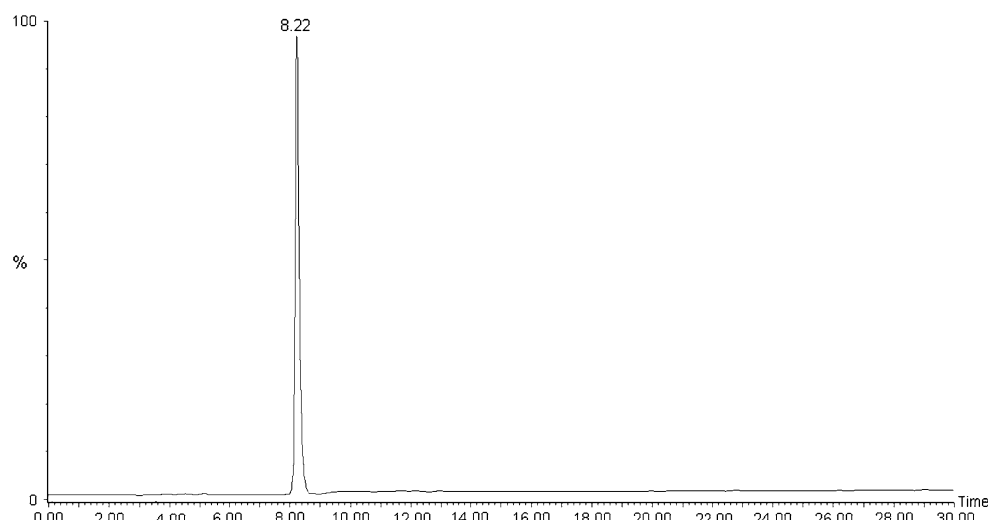


Fig. 3 ESI-MS of the purified analog Ser-Val-Asp-Gln-Arg-Gly-Asn-Gln-NH₂

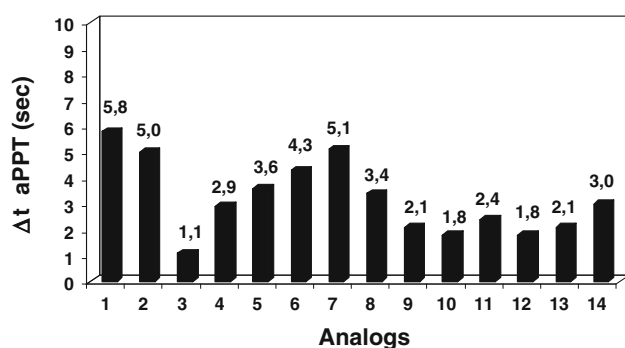
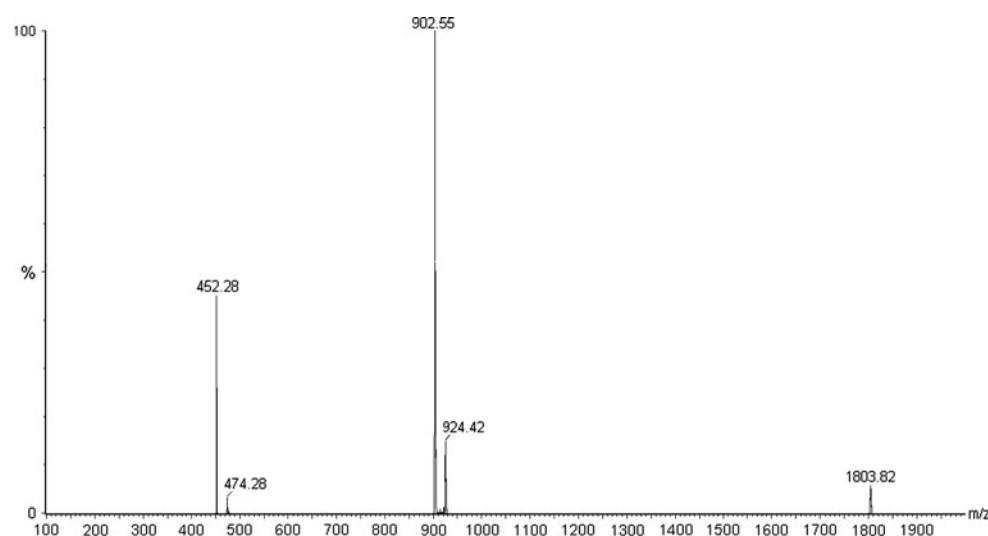


Fig. 4 Divergence of activated partial thromboplastin time ($\Delta t = \text{aPTT}_{\text{sample}} - \text{aPTT}_{\text{control}}$)

However, it seems interesting that the replacement of Asn⁵⁶⁴ with Glu (6) enhances the inhibitory activity in comparison with the native peptide, whereas substitution with Asp in the same position (2) slightly reduces this activity. Substitution of Asn⁵⁶⁴ by Gln and acetylation at

N^α-terminal site (12) yields an interesting result too. On the other hand, transformation of peptide 1 to the C-terminal carboxamide (7) decreases the inhibitory activity of the peptide. N^α-acetylation of the above peptide (14) results in a significant loss of the activity. Similar result is obtained by the N^α-acetylated analog of peptide 6, resultant the analog 13. Analogs 8 and 9, N^α-acetyl products of analogs 1 and 2, respectively, show satisfactory activity too. It is also clear that those modifications by introduction of Asp β-alkylated products at the position of Asn⁵⁶⁴ (3, 4, 10, and 11) show a rather insufficient activity.

Variations of the results between the two anticoagulant assays are rather expected as the aPTT assay is a general screening test, while monitoring of FVIII activity assay is a more specific test. These variations have to be investigated by examining the interaction of these peptides with other blood coagulation factors involved in the extrinsic pathway.

Considering all the above mentioned remarks, we can conclude that: (i) analogs of the sequence Ser⁵⁵⁸-Gln⁵⁶⁵ of

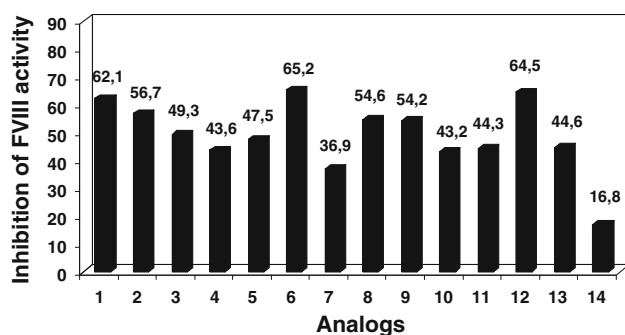


Fig. 5 Inhibition (%) of the FVIIIa activity caused by the synthesized analogs. Inhibition (%) of the FVIIIa activity = $[(100\% \text{ value FVIIIa activity}_{\text{control}} - \% \text{ value FVIIIa activity}_{\text{sample}}) / \% \text{ value FVIIIa activity}_{\text{control}}] \times 100\%$

the A2 subunit of FVIIIa that interacts with FIXa represent a promising epitope, acting as intrinsic anticoagulants targeting FVIII–FIX interaction; (ii) modifications at the position 564, according to the biological results, show that Asn⁵⁶⁴ is rather a crucial residue for its anticoagulant activity; (iii) both anticoagulant assays (general screening test as well as more specific assays, monitor of FVIII or other factors activity) are essential for studying the role of these peptides.

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