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ORIGINAL ARTICLE

Cyclic peptide analogs of 558–565 epitope of A2 subunit of Factor VIII prolong aPTT. Toward a novel synthesis of anticoagulants

C. Anastasopoulos · Y. Sarigiannis · G. Stavropoulos

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Abstract Novel anticoagulant therapies target specific clotting factors in blood coagulation cascade. Inhibition of the blood coagulation through Factor VIII–Factor IX interaction represents an attractive approach for the treatment and prevention of diseases caused by thrombosis. Our research efforts are continued by the synthesis and biological evaluation of cyclic, head to tail peptides, analogs of the 558–565 sequence of the A2 subunit of FVIII, aiming at the efficient inhibition of Factor VIIIa–Factor IXa interaction. The analogs were synthesized on solid phase using the acid labile 2-chlorotrityl chloride resin, while their anticoagulant activities were examined in vitro by monitoring activated partial thromboplastin time and the inhibition of Factor VIII activity. The results reveal that these peptides provide bases for the development of new anticoagulant agents.

Keywords Factor VIII · Anticoagulant activity · Activated partial thromboplastin time · Synthetic cyclic peptides · Peptoid-peptides · FVIII–FIX interaction · Antithrombotic agents

Abbreviations

AcOH	Acetic acid
aPTT	Activated partial thromboplastin time
Boc	*tert*-Butoxycarbonyl
Bzl	Benzyl group
CLTR-Cl	2-Chlorotrityl chloride resin
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
PPP	Platelet poor plasma
PyBOP	Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
PT	Prothrombin time
rFVIII	Recombinant factor VIII
Bu^t	*tert*-Butyl group
RP-HPLC	Reversed-phase high-performance liquid chromatography
TBTU	2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethylammonium tetrafluoroborate
TES	Triethylsilane
TFA	Trifluoroacetic acid
TFE	2,2,2-Trifluoroethanol
TLC	Thin layer chromatography
Tol	Toluene
Trt	Trityl, triphenylmethyl group
vWF	Von Willebrand factor

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, *Amino Acids*, Pept. Proteins, (2012) 37, xi–xviii.

C. Anastasopoulos · Y. Sarigiannis · G. Stavropoulos (✉)
Department of Chemistry, University of Patras, Patras, Greece
e-mail: G.Stavropoulos@chemistry.upatras.gr

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Introduction

Anticoagulant therapy is a mainstay in medical practice to cope with the cardiovascular diseases (CVDs), such as deep vein thrombosis, myocardial infarction, unstable angina, pulmonary embolism and ischemic stroke. The current anticoagulant therapies cover a range of Vitamin K antagonists, heparins, direct thrombin or Factor Xa inhibitors. Although these drugs are effective, they have numerous limitations. Most of them have a slow onset of action, a variable dose requirement due to common genetic polymorphisms (e.g., warfarin), food-drug or drug–drug interactions, etc. Because of these issues, routine patient monitoring is essential to ensure that a therapeutic anticoagulation response is obtained. Newer anticoagulants have many potential advantages; however, there are some potential disadvantages, particularly the lack of established monitoring tests, therapeutic ranges and reversal agents (Witt and Clark 2013; Maan et al. 2012). In addition, at least some of the extended research studies in orthopedic knee or hip surgery suggest that there will continue to be a trade-off (Garcia et al. 2010). After all, it is clear that there is a continuous requirement in targeting new directions to succeed further development of new and safer antithrombotic drugs without the above limitations. These include inhibitors of the factors VIIa, Xa, IXa, VIIIa and XII (Lin et al. 2006; Minors 2007; Howard et al. 2007; Franchini and Mannucci 2011; Baeriswyl et al. 2013).

Haemostasis basics Upon vessel injury, the platelets adhere to macromolecules in subendothelial tissues at the site of injury and then aggregate to form the primary hemostatic plug. Coagulation, one of the most important host defense mechanism, is a complex protease cascade involving about 30 interacting proteins. The result is the formation of thrombin, which catalyzes the conversion of fibrinogen, a soluble protein, to insoluble fibrin strands, which forms a stable clot in conjunction with platelets. This cascade follows two biochemical pathways initiated by either the exposure of tissue factor (TF) on the vessel wall at the site of injury, "extrinsic pathway", or by the activation of blood-borne components (FXII) by biologic or foreign negatively charged surfaces, "intrinsic pathway". Recently, new evidences (Gailani and Renne 2007; Woodruff et al. 2010) indicate the important participation of the intrinsic pathway in sustaining thrombus development in vivo. A heterotrimer complex, called *tenase*, is formed among FVIIIa, FIXa, Ca^{2+} (which come from activated platelets) and negatively charged phospholipids of the cells membrane. *Tenase* is responsible for the conversion of Factor X to the activated form FXa, another protease that binds to cofactor Va to form a complex known as *prothrombinase*. The latter complex converts prothrombin to thrombin, which acts on fibrinogen to generate a fibrin monomer, polymerized rapidly to form fibrin clot.

Factor VIII is a multidomain glycoprotein responsible for blood coagulation pathway. This is constituted of six domains, which can be schematically represented as followed: A1–A2–B–A3–C1–C2 (Vehar et al. 1984). FVIII circulates in blood mainly as a heterodimer composed of a heavy chain (A1–A2–B) and a light chain (A3–C1–C2) complexed to Von Willebrand factor (vWF) associated with a lipoprotein receptor-related protein (LRP) (Fay 2004). The main role of vWF is the engagement in other proteins, particularly in factor VIII and consequently it is important in the adherence of platelets at the area 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). Among the FVIII six domains, the A2 one is a key element that regulates its overall function. In addition, the A2 domain is of primary importance in the cofactor function of FVIII, because it contains FIXa binding sites (Fay et al. 1994; O'Brien et al. 1995; Bajaj et al. 2001). The A2 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 (Shen et al. 2008; Ngo et al. 2008; Jagannathan et al. 2009). Recently, Plantier et al. (2012) provided a detailed map of the FVIII A2 domain between residues 371 and 649, identifying residues crucial for maintaining FVIII function and residues that can be mutated without jeopardizing the coagulant activity of the factor. Moreover, Griffiths et al. (2013) demonstrated a previously unidentified high affinity interaction site between the FVIIIa A2 subunit and FIXa, region 707–714.

We published recently (Anastasopoulos et al. 2013) that small synthetic linear peptides, analogs of the sequence 558–565 of the A2 subunit, might be used as lead compounds for novel anticoagulant drugs by inhibiting the FVIIIa–FIXa interaction. Linear peptides that contain 2–10 residues, as in the case of 558–565 loop, are especially flexible in solution. Their geometry and especially their flexibility can be modified by the synthesis of cyclic peptides using a wide variety of classical and novel chemical methods (Perlman et al. 2005; Thakkar et al. 2013). Usually, their constrained structure results in higher receptor-binding affinities as compared to their linear analogs. Moreover, cyclization of peptides usually enhances their resistance to enzymatic degradation by proteases, so that could prolong their biological activity. Considering all the above-mentioned remarks, we synthesized a series of cyclic peptides (head to tail), analogs of the above sequence of A2 aiming at preventing the interaction of FVIIIa with FIXa, to suspend the process of blood

coagulation, in vitro. In addition, a series of peptide–peptoid hybrids (*peptomers*) containing both peptide and *N*-substituted glycine residues have been synthesized and tested for their anticoagulant activity (Vakalopoulou et al. 2005).

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), 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 MassLynx 4.1 software. Cone voltage was set at 30 V and scan time at 1 s, with interscan delay at 0.1 s.

Synthesis

Anchoring on the resin

The examined peptides were synthesized using CLTR-Cl by Fmoc solid-phase peptide synthesis protocol. The esterification of the first Fmoc-amino acid onto the CLTR-Cl was carried out as firstly 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 gently stirred 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 × 5 mL × 10 min) at RT. The Fmoc-amino acid resin was filtered and washed successively with DCM (3 × 5 mL), DMF (3 × 5 mL), 2-propanol (3 × 5 mL) and *n*-hexane (1 × 5 mL) and dried over P_2O_5 under vacuum. Subsequently, the Fmoc group was removed from the Fmoc-amino acid resin by treatment with a solution of 25 % 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/H_2O (5:1), $CHCl_3$/MeOH/AcOH (85:10:5) or Tol/MeOH/AcOH (7:1.5:1.5) as development solvents. (The TLC procedure has as following: 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 firstly dried and visualized under UV lamp at 254 nm followed by ninhydrin spraying. In case of uncompleted reaction, a magenta spot near the baseline of TLC was observed. The Fmoc group deprotection was performed as described above, using 20 % piperidine in DMF for 30 min, while the amino acid side-chain protection was as follows: Me, Bzl and Bu^t for Asp; Bu^t for Ser, Pbf for Arg, Trt for Gln and Asn. All coupling and deprotection steps have been repeated until the desired sequence to be completed.

Cleavage from the resin and cyclization

The synthesized protected peptide was cleaved from the resin in the next step. The peptide–resin ester was cleaved with a mixture of DCM/TFE (70:30) for 2 h at RT. The resin was then filtered off, and the solvent was removed using a rotary evaporator. The obtained crude oily product (protected peptide) was washed with DCM and precipitated by the addition of cold anhydrous diethyl ether as a white solid. The cyclization of the linear protected peptides was achieved using standard reagents and procedures such as HOBt (4 equiv of the linear protected analog), TBTU (4 equiv of the linear protected analog) and collidine (6 equiv of the linear protected analog) reacting for ~48 h in DMF (0.05 mmol of the linear protected analog/100 mL). The side deprotection (groups Bu^t, Pbf and Trt) of the cyclic protected peptides was achieved using a mixture of TFA/DCM/TES (90:5:5) and gently stirred for 4 h at RT. The solvent was evaporated *under vacuum*, the crude free cyclic peptide was precipitated with cold anhydrous diethyl ether and collected by filtration (Fig. 1).

Purification and characterization of the crude peptides

The crude linear protected intermediate octapeptides 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 (250 mm × 10 mm, 7 μm) by gradient elution of 5–85 % solvent B (solvent A: 0.1 % TFA in H_2O; solvent B: 0.1 % TFA in MeCN) over 70 min at a flow rate of 2.5 mL/min at 214 nm and 254 nm. All

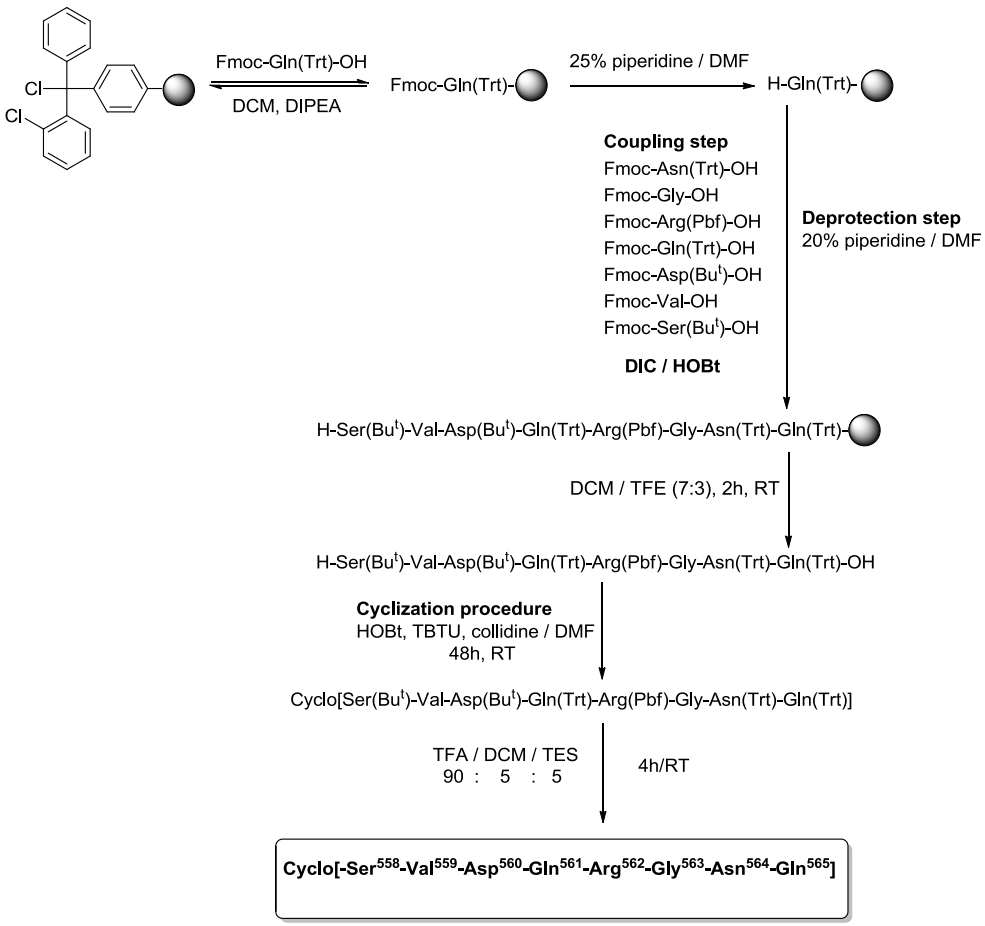


Fig. 1 Synthetic procedure of cyclic analogs of the sequence Ser^{558}-Gln^{565}

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 nm and 254 nm. Separation of the final deprotected peptides 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.

General route for the synthesis of the cyclic peptidomimetics

The linear protected peptidomimetics were synthesized on CLTR-Cl by following the procedure described above with some modifications (Vakalopoulou et al. 2005) as firstly depicted in the submonomer process (Zuckermann et al. 1992). The amino terminus of the growing peptide chain was firstly bromoacetylated. Afterwards it was reacted with benzylamine to yield the corresponding *N*-substituted glycine residue, [–N(CH_2–Ph)–CH_2–CO–], (NPhe). Briefly, after the anchoring of the two first amino acids of the desired sequence, Br–CH_2–COOH (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 the coupling was verified by the Kaiser's test and TLC, using MeCN/H_2O (5:1), $CHCl_3$/MeOH/AcOH (85:10:5) or Tol/MeOH/AcOH (7:1.5:1.5) as development solvents. In case of uncompleted reaction, a magenta spot near the baseline of TLC was observed. Subsequently, benzylamine (9 equiv of the resin substitution) dissolved in DMF was added and the mixture was left to react for 24 h. Progress and completeness of the coupling was verified by TLC, as described above, and HPLC. The other remaining protected amino acids for the elongation of the chain were coupled as previously described using DIC/HOBt as coupling reagents. Cleavage of the peptidomimetics from the resin and cyclization was followed using the same reagents and method as described previously for the peptides. Then, the side deprotection of the cyclic protected hybrids peptoid–peptides was achieved using a mixture of TFA/DCM/TES (90:5:5) and gently stirred for 3 h at RT. The solvent was evaporated *under vacuum*, the crude free cyclic peptidomimetics were

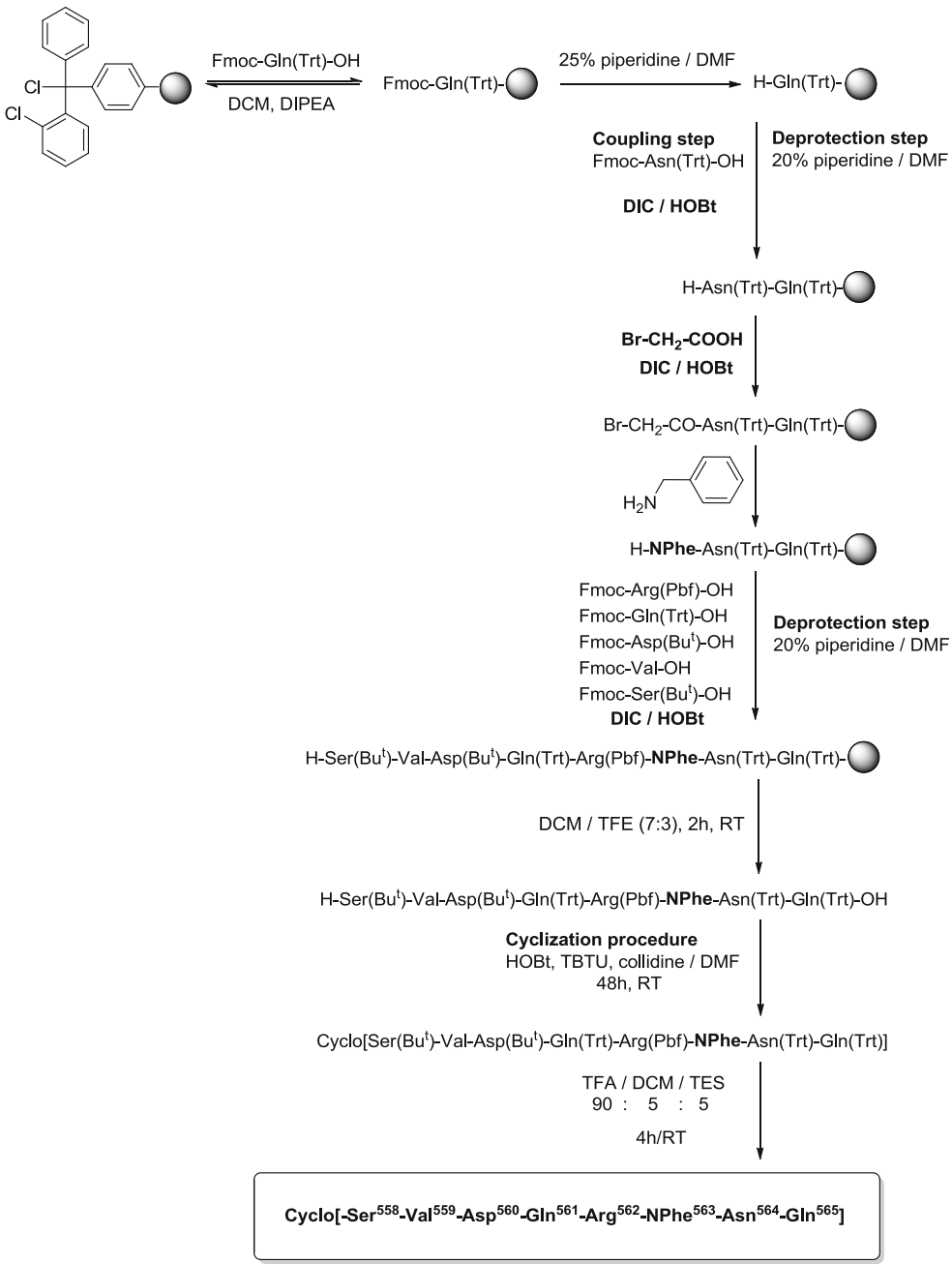


Fig. 2 Synthetic procedure of cyclic peptides-peptoids of the sequence Ser^{558}-Gln^{565}

precipitated with cold anhydrous diethyl ether and collected by filtration. Further purification and characterization of the synthetic peptidomimetics were achieved 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 (250 mm × 10 mm, 7 μm) by gradient elution of 5–85 % solvent B (solvent A: 0.1 % TFA in H_2O; solvent B: 0.1 % TFA in MeCN) over 70 min at a flow rate of 2.5 mL/min at 214 nm 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 (Fig. 2).

Biological-anticoagulant assays

Measurements were performed on an automatic analyzer ACL Elite Pro; whereas haemostasis 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 RT to obtain platelet poor plasma (PPP). Citrated samples can be run within 2 h of sample collection.

Measurement of activated partial thromboplastin time (aPTT) assay

An aliquot of PPP (200 μL) was transferred into a vial, and peptide solution (200 μL, 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 μL) and buffer (200 μL) and was incubated under the same conditions. After that, the incubated peptide solution (10 μL) was transferred to the instrument cuvette as well as cephalin–kaolin solution (10 μL, 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 μL, 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 the 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

When factor VIII is added to plasma containing a FVIII antagonist and the mixture is incubated, then the FVIII will be 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 μL, diluted (1:1) in Owren–Koller buffer pH 7.4, 1 U/mL) was transferred into a vial containing peptide solution (200 μL, 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 μL, diluted (1:1) in Owren–Koller buffer pH 7.4, 1 U/mL], whereas Control 2 contained rFVIIIa [200 μL, diluted (1:1) in Owren–Koller buffer pH 7.4, 1 U/mL] and buffer (200 μL). All the sample vials were incubated at 37 °C for 30 min. Then, the incubated peptide solution (10 μL) was transferred to the instrument cuvette as well as cephalin–kaolin solution (10 μL), acting as aPTT contact activator and dPPP (deficient PPP to FVIII, 10 μL). The cuvette was transferred to the measuring position, and $CaCl_2$ (25 mM, 10 μL) was added to prolong further reactions. Inhibition values were reported and extracted automatically by the instrument (Barrowcliffe et al. 2002).

Results and discussion

Anticoagulants are effective agents used widely for the prevention and treatment of venous thromboembolism. Despite their clinical efficiency, traditional anticoagulants are all associated with significant drawbacks (slow onset of action, routine blood PT and aPTT monitoring, bleeding complications, interactions with other drugs, dietary implications) (Gailani and Renne 2007). As a result, modulation of the coagulation process represents an important target in the development of new oral and parenteral anticoagulants today (Haas et al. 2012; Witt and Clark 2013). Interactions between blood coagulation proteases such as FVIIIa–FIXa have captured scientific interest during the last decade (Woodruff et al. 2010; Maan et al. 2012; Jagannathan et al. 2009).

We have recently shown that synthetic linear peptides (Anastasopoulos et al. 2012, 2013), analogs of the sequence Ser^{558}-Gln^{565} of the A2 subunit of FVIIIa represent a promising epitope, acting as intrinsic anticoagulant targeting the FVIIIa–FIXa interaction.

Ser^{558}-Val^{559}-Asp^{560}-$Gln^{561}Arg^{562}$-Gly^{563}-Asn^{564}-Gln^{565}

By continuing this research effort, we present here a series of cyclic synthetic peptides and peptidomimetics, analogs of the linear peptides mentioned above. In most cases, Asn^{564} has been replaced by Gln, Glu, Asp and β-benzyl aspartate, whereas Arg^{562} was replaced by Lys. Peptide–peptoid hybrids, *peptomers*, that are peptides containing *N*-substituted glycine residues, have also shown significant utility (Ostergaard and Holm 1997) in the drug discovery and development. Thus, Gly^{563} has been replaced by NPhe, constructing a linear hybrid peptide–peptoid aiming to synthesize a molecule with improved stability to proteolytic enzymes, bioavailability and similar anticoagulant effect as the native linear peptide (Anastasopoulos et al. 2010).

All the analogs presented in Table 1 were synthesized manually using as solid support, the 2-chlorotrityl chloride resin and standard coupling procedures for Fmoc/Bu^t methodology. This type of resin as well as the efficient protection of the side chain of Gln residue with Trt group yielded final products with high purity. In addition, the application of well-established methods and procedures for the head to tail cyclization eliminated the cyclization

Table 1 Analytical data of the synthesized cyclic peptides

	Synthesized cyclic analogs	M_{calcd}	M_{found}	t_R (min)
I	Cyclo[-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-**Asn^{564}**-Gln^{565}]	884.9	885.8	12.35 (i)
II	Cyclo[-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-**Asp^{564}**-Gln^{565}]	885.9	886.8	11.73 (i)
III	Cyclo[-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-**Asp(OBzl)^{564}**-Gln^{565}]	976.0	976.9	18.28 (i)
IV	Cyclo[-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-**Gln^{564}**-Gln^{565}]	898.9	899.5	14.79 (i)
V	Cyclo[-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-Gly^{563}-**Glu^{564}**-Gln^{565}]	899.9	900.4	14.32 (i)
VI	Cyclo[-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-**Lys^{562}**-Gly^{563}-**Asp^{564}**-Gln^{565}]	857.9	858.5	12.99 (i)
VII	Cyclo[-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-**NPhe^{563}**-Asn^{564}-Gln^{565}]	975.0	975.4	17.80 (ii)
VIII	Cyclo[-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-**NPhe^{563}-Asp^{564}**-Gln^{565}]	976.0	976.4	13.95 (ii)
IX	Cyclo[-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-**NPhe^{563}-Asp(OBzl)^{564}**-Gln^{565}]	1,066.1	1,066.7	24.58 (ii)
X	Cyclo[-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-Arg^{562}-**NPhe^{563}-Glu^{564}**-Gln^{565}]	990.0	990.5	18.98 (ii)
XI	Cyclo[-Ser^{558}-Val^{559}-Asp^{560}-**Asp^{561}**-Arg^{562}-**NPhe^{563}-Gln^{564}**-Gln^{565}]	976.0	976.6	18.69 (ii)

(i), Linear gradient 0–30 % B for 30 min; (ii), linear gradient from 0–40 % B for 30 min (A: 0.1 % TFA in water, B: 0.1 % TFA in MeCN)

byproducts, thus no dimers or oligomers formation was observed. Moreover, no racemization was noted although the cyclization took place among Gln^{565} and Ser^{558}, possibly due to their protecting groups. The cyclization yield was estimated from the peak abundance, considering the different retention times of linear and cyclic peptides. 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 are shown in Figs. 3 and 4, respectively, for the analog **VI**, Cyclo[-Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}-**Lys^{562}**-Gly^{563}-**Asp^{564}**-Gln^{565}].

The synthesized compounds were tested for their anticoagulant activity by measuring their aPTT and PT assay, a global screen clot-based method for the evaluation of the intrinsic, extrinsic and common coagulation pathways (Samama and Guinet 2011). Clot-based tests measure the time taken for plasma to clot after the addition of calcium ions and an activator. Clot formation changes the light transmittance, which is converted through a microcomputer as determined coagulation time. PT and aPTT tests are the routine blood-monitoring tests when a coagulation disorder is suspected, current anticoagulant treatment with warfarin or heparins is applied, as well as for a preoperative screening (Chitlur 2012).

A short aPTT is associated with a high risk of venous thromboembolism, while prolonged clotting time is observed in case of factor deficiencies, liver disease, vitamin K deficiency, heparin presence or other factor inhibitors (CLSI 2008). A prolonged aPTT with normal PT indicates a specific or multiple factor (VIII, IX, XI and XII) deficiency, the presence of a factor-specific inhibitor, or the presence of a nonspecific 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.

All the tested peptides or peptidomimetics showed normal PT as it was expected; abnormal PT is associated with deficiencies of extrinsic pathways factors (V, VII and X factor) and prothrombin or fibrinogen disorders.

Figure 5 proves the effect of the synthesized compounds on the aPTT assay. Clearly, the cyclization of the native linear sequence 558–565 has no significant effect on the prolongation of coagulation time.

On the contrary with the linear peptide (**C**) which shows a divergence of coagulation time of 5.8 s (Anastasopoulos et al. 2013), the cyclic compound (**I**) loses dramatically its activity resulting in a very short Δt aPTT, only 0.9 s. Moreover, the same phenomenon is observed with the cyclic analog (**II**) and its linear peptide that has incorporated Asp instead of Asn^{564}. Incorporation in the sequence of Lys instead of Arg^{562} and Asp instead of Asn^{564} (**VI**), produces a cyclic analog with very strong activity. Thus, the resultant analog **VI** reveals an impressive Δt aPTT (16.1 s), longer than all the other synthesized compounds present, even longer than the linear sequence of the native peptide. Possibly, this cyclic peptide shows higher activity not only due to its resistance to enzymatic degradation, but also to a further constrained structure due to the sequence -Asp^{560}-Gln^{561}-Lys^{562}-Gly^{563}-Asp^{564}-, which could lead in salt bridge formation between Lys and the neighborly Asp units. In addition, in comparison with its corresponding linear peptide which shows a very short Δt aPTT (1.5 s), (Anastasopoulos et al. 2012), cyclization enhances strongly the anticoagulant activity. All other substitutions of Asn^{564} do not prolong notably the aPTT, (Table 2). In addition, the replacement of Gly^{563} by NPhe, resulting in the synthesis of *peptomers* (Ostergaard and Holm 1997), does not give any significant

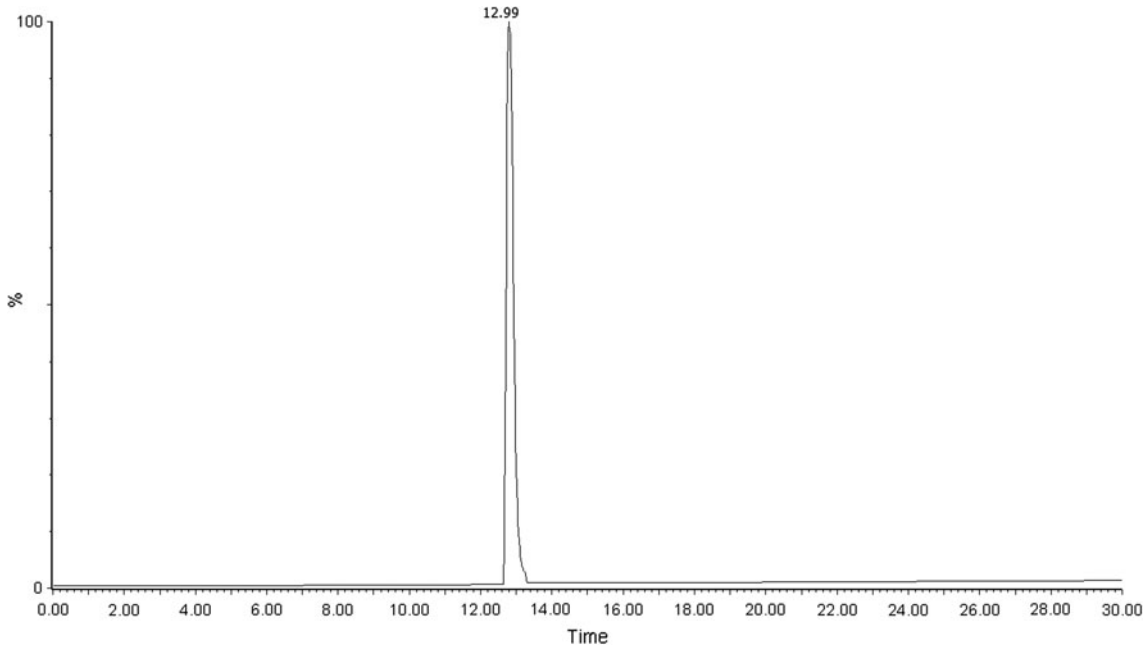
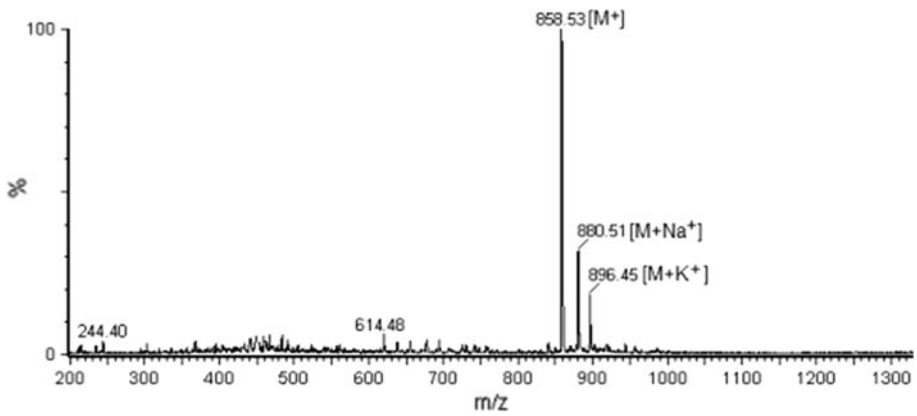


Fig. 3 RP-HPLC of the final purified analog **VI**

Fig. 4 ESI–MS of the final purified analog **VI**



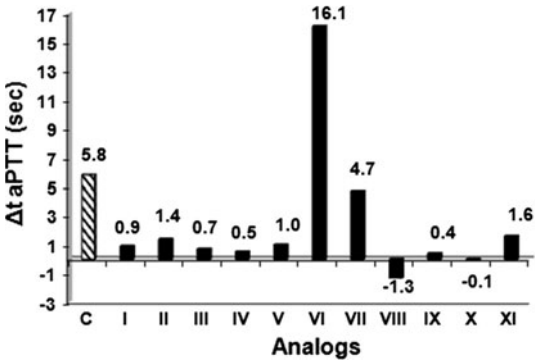


Fig. 5 Divergence of activated partial thromboplastin time ($\Delta t = aPTT_{sample} - aPTT_{control}$). *C* Control: native linear peptide: Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}Arg^{562}-Gly^{563}-Asn^{564}-Gln^{565}

Table 2 The divergence of activated partial thromboplastin time (Δt aPTT) of the cyclic peptides (**I–VI**) and their corresponding linear ones

	Synthesized analogs of the 558–565 linear sequence[a]	Δt aPTT of cyclic analogs	Δt aPTT of linear analogs
I	-Asn^{564}-	0.9	5.8
II	-Asp^{564}-	1.4	5.0
III	-Asp(OBzl)^{564}-	0.7	2.9
IV	-Gln^{564}-	0.5	3.6
V	-Glu^{564}-	1.0	4.3
VI	-Lys^{562}-, -Asp^{564}-	16.1	1.5

[a] Ser^{558}-Val^{559}-Asp^{560}-Gln^{561}Arg^{562}-Gly^{563}-Asn^{564}-Gln^{565}

prolongation of aPTT. Only the cyclic analog **VII** displays a Δt aPTT similar with the native peptide sequence (**C**) and the linear peptomer (Anastasopoulos et al. 2010). In contrast, the analogs **VIII** and **X** accelerate the clot formation, indicative that they enhance FVIIIa–FIXa complex formation.

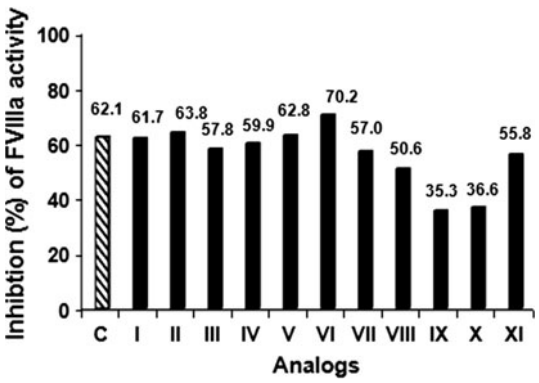


Fig. 6 Inhibition (%) of the FVIIIa activity caused by the synthesized analogs. *C* Control: native linear peptide: Ser^{558}-Val^{559}-Asp^{560}-$Gln^{561}Arg^{562}$-Gly^{563}-Asn^{564}-Gln^{565}. Inhibition (%) of the FVIIIa activity = [(100 % value FVIIIa $activity_{control}$ − % value FVIIIa $activity_{sample}$)/% value FVIIIa $activity_{control}$] × 100 %

Another useful and more specific test for monitoring hypercoagulable state is Factor VIII activity. We have examined the influence of these synthetic cyclic peptides and peptidomimetics 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 firstly. Then, rFVIII is added to dPPP containing a synthetic peptide and the mixture incubated at 37 °C for 30 min. A reduced FVIIIa activity is observed when a synthetic peptide acts as inhibitor.

The Fig. 6 depicts the % inhibition of the FVIIIa activity due to the presence of the synthesized analogs. Comparing the cyclic analog **I** with the native linear sequence (**C**), we ascertain that cyclization of the linear peptide does not affect at all the inhibition of FVIIIa activity. On the contrary, cyclic analogs **II**, **III**, **IV**, inhibit FVIIIa activity higher than their corresponding linear ones. Analog **VI** (replacement of Arg^{562} with Lys and Asn^{564} with Asp) causes the highest % inhibition of FVIIIa activity of all the synthesized compounds. In comparison with its linear peptide % inhibition (47.9 %) (Anastasopoulos et al. 2012), **VI** inhibits strongly FVIIIa activity (70.2 %). Moreover, it causes even higher % inhibition of FVIIIa activity in comparison with analog **I** (61.7 %) and the native linear peptide (**C**) (62.1 %). On the other hand, incorporation of NPhe, instead of Gly^{563}, does not have any positive impact on the inhibition of FVIIIa activity. Nevertheless, regarding the peptomers, it is notable that any substitution of Asn^{564} with Asp, β-benzyl aspartate, Glu or Gln results in a considerable loss of activity. Particularly, analogs **IX** and **X** exhibit very little inhibition of FVIIIa activity, 35.3 and 36.6 %, respectively. By comparing the results of peptomers **VIII**, **IX** and **X**, apparently, masking of the aspartic acid side chain with benzyl group or a small elongation of it ($–CH_2–$) affects notably the inhibition of FVIIIa activity. Therefore, in case of peptomers, side-chain length and charge at the position 564 seem to be important for the inhibition of FVIIIa activity, while this phenomenon is not observed in case of cyclic peptides **I** to **V**.

Considering all the above-mentioned remarks, we can conclude that:

1. Head to tail cyclization of linear peptides with a backbone of eight amino acids is remarkably efficient either for peptides or peptide–peptoid hybrids (peptomers).
2. The cyclic peptides II, III, IV and V showed higher inhibition activity than the corresponding linear ones.
3. The replacement of Arg^{562} with Lys and Asn^{564} with Asp (**VI**), results in a promising candidate molecule, acting as intrinsic anticoagulant targeting FVIIIa–FIXa interaction. In addition, there is no interference with the extrinsic cascade implicated in physiological coagulation. Further investigation on the backbone properties is essential to improve its ability to interfere more effectively at FVIII–FIX interaction.
4. Incorporation of an *N*-substituted Gly (NPhe) residue in the peptide chain does not improve the anticoagulant activity of the cyclic native peptide (II).

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Conflict of interest The authors declare that they have no conflict of interest.

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