Journal of Peptide Science
J. Pept. Sci. 2008; **14**: 946–953
Published online 28 March 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.1030

JOURNAL OF PEPTIDE SCIENCE

Peptides and pseudopeptides incorporating D-Phe–Pro–Arg and Arg–Gly–Asp lead sequences as potential antithrombotic agents

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Received 12 November 2007; Revised 11 January 2008; Accepted 30 January 2008

Abstract: Peptide leads D-Phe–Pro–Arg for thrombin inhibition and Arg–Gly–Asp for antagonistic activity on fibrinogen receptor were combined in one molecule in order to produce compounds capable of acting both as thrombin inhibitors and as fibrinogen receptor antagonists. Peptide conjugate **7** possessing both leads joined by a tetraglycine linker as well as tripeptides and peptidomimetics with highly overlapped D-Phe–Pro–Arg and Arg–Gly–Asp pharmacophore groups were prepared. Conjugate **7** was found to possess antagonistic activity on fibrinogen receptor, but was unexpectedly inactive as thrombin inhibitor. Compound **9** comprising of highly integrated D-Phe–Pro–Arg and Arg–Gly–Asp pharmacophore groups was found to possess a moderate but well balanced thrombin inhibitory and fibrinogen receptor antagonistic activity. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: dual activity; fibrinogen receptor antagonists; peptide conjugate; peptidomimetic; thrombin inhibition

INTRODUCTION

Thromboembolic diseases are a leading cause of mortality and morbidity in the developed world [1]. Their successful treatment thus has a very important effect on the population health and also has a major economic impact on society. Developing drugs which would successfully treat thromboembolic diseases is therefore a prominent goal of medicinal chemistry. Antithrombotic agents can be classified into anticoagulants, antiplatelet agents, and fibrinolytic drugs [2]. The anticoagulants act either by modulating the endogenous levels of the key coagulation enzymes such as thrombin, factor Xa and factor VIIa or by inactivating their enzymatic activity. Antiplatelet agents inhibit platelet activation and aggregation, a crucial process of hemostasis and thrombus formation. Fibrinolytic agents, which are administered intravenously under clinical emergency, exert their action by lysis of existing blood clots and restore blood flow in occluded vessels. The main research activity in the field of discovery of new antithrombotic agents is devoted to new anticoagulants and antiplatelet drugs. Although it seemed that a major breakthrough in anticoagulant therapy was achieved by introduction of thrombin inhibitor argatroban [3], it did not achieve a widespread use, and further development of thrombin inhibitors brought some bitter disappointments, e.g. the withdrawal of recently introduced ximelagatran [4]. Thus, development of effective and patient friendly antithrombotic agents remains a permanent challenge to medicinal chemists.

To achieve an efficient antithrombotic effect, a combination of various antiaggregatory and anticoagulant agents is frequently used in clinical practice. The combined use of anticoagulant and antiplatelet drugs has an additive effect by suppressing both blood coagulation and platelet function and is thus more effective than treatment directed against thrombin or platelets alone [5,6]. Therefore, the therapeutic potential of combining antiaggregatory and anticoagulant activity in a single compound has now been realized.

Several chimeric peptides with anticoagulant and antiplatelet aggregation activities have been engineered from hirudin – a natural inhibitor of thrombin from medicinal leach (*Hirudo medicinalis*) [7]. With the thrombin inhibition constant (K_i) in femtomolar range hirudin earlier attracted attention of medicinal chemists and many modifications of its structure were performed. Hirudin occupies with its *C*-terminus the thrombin anion-binding exosite (ABE) and covers with its *N*-terminal part the catalytic site, while other thrombin inhibitors being mainly D-Phe–Pro–Arg mimetics, bind to the active site. Conjugates possessing D-Phe–Pro–Arg motif and the hirudin *C*-terminal sequence, which would simultaneously occupy both binding sites on the target protein, were synthesized. Comparison showed that such conjugates named *hirulogs*, had better anticoagulant activity than hirudin itself [8]. In *hirudisins*, hirudin-derived thrombin inhibitors with disintegrin activity, Ser–Asp–Gly–Glu sequence (residues 32–35) of hirudin was replaced with

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integrin-specific Arg–Gly–Asp–Ser (RGDS) sequence to gain antiplatelet aggregation activity beside anticoagulant activity [9]. Similarly, a chimeric peptide consisting of the RGDS sequence followed by the carboxyl-terminal region of hirudin (residues 53–64) was synthesized which also exhibited both antiaggregatory and antithrombin activities [10]. Trifunctional antithrombin and antiplatelet peptide with dual mode of action was obtained by coupling a thrombin catalytic site inhibitor *D*-Phe–Pro–Arg with an ABE associating moiety (hirudin$_{55-65}$ sequence) via a bridging sequence containing RGD motif [11].

Although several attempts have been described to design chimeric peptides with antithrombin and antiplatelet aggregation activities, no work was done to combine peptide leads *D*-Phe–Pro–Arg and Arg–Gly–Asp in one molecule in order to produce compounds capable of acting both as thrombin inhibitors and as fibrinogen receptor antagonists. The aim of the present work was to design and synthesize peptidic multiple ligands incorporating RGD and fPR peptide leads, which according to the designed multiple ligand paradigm [12] will be joined with a peptide linker or be overlapped. Additionally, tripeptides resulting from this approach will be modified by isosteric replacements with amino acid surrogates to give peptidomimetics.

MATERIALS AND METHODS

Chemicals and Equipment

Solvents and reagents, unless otherwise stated, were of analytical grade, and were obtained from Reanal Fine Chemical Works (Budapest, Hungary). *N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (water soluble carbodiimide (WSC)), diisopropylcarbodiimide (DIC), *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyl-uronium hexafluorophosphate (HATU), 1-hydroxybenzotriazole (HOBt), diisopropyl ethylamine (DIEA), ethanedithiol (EDT), triisopeoylsilane (TIS) and piperidine were purchased from Fluka (Hungary). Fmoc-amino acids, Rink amide 4-methylbenzhydrylamine (MBHA) and Wang resins were obtained from Novabiochem (Merck Kft, Budapest, Hungary). Fmoc amino acid chlorides were prepared from corresponding Fmoc amino acids as described in Ref. 13. Analytical RP-HPLC was run on a Knauer (Bad Homburg, Germany) instrument with a Phenomenex Jupiter (C18, 5 μm, 300 Å, 250 × 4.6 mm) column, using a linear gradient with eluents A [0.1% TFA in water] and B [0.08% TFA in acetonitrile] at flow rate 1 ml/min. Peaks were detected at $\lambda = 220$ nm. Products were purified using semipreparative HPLC Knauer instrument with a Phenomenex Jupiter (C18, 5 μm, 300 Å, 250 × 10 mm) column at flow rate 4 ml/min or FPLC instrument (Pharmacia, Uppsala, Sweden) with a Vxydac (C18, 300 Å, 25 μm, 480 × 25 mm) column at flow rate 8 ml/min. Electrospray ionization mass spectrometry was performed on a Bruker Esquire 3000+ ion trap mass spectrometer (Bremen, Germany). Electrospray ionization high resolution mass spectrometry (HRMS) was performed using a Waters (Micromass) Q-TOF Premier mass spectrometer (Milford, USA).

Peptide Synthesis

Peptides were synthesized manually on solid phase using Fmoc technique. Coupling of amino acid (3 equiv.) to free amino group was performed using DIC (3 equiv.) and HOBt (3 equiv.). Coupling typically lasted 1 h and was monitored by Ninhydrin (Kaiser test) [14]. Level of first amino acid attachment was determined spectrophotometrically at 405 nm [15]. The standard protocol for cleavage of Fmoc protecting group consisted of two treatments (2 and 18 min) with 20% piperidine in DMF. All peptides unless otherwise stated were obtained as trifluoroacetate salts. For MS, HRMS and HPLC data of the peptides see Table 1.

Synthesis of Cyclic Hexapeptide 2

Solid phase synthesis. H–Cys–Arg(Tos)–Gly–Asp(OcHex)–Cys–Gly–NH_2 (**1**) was synthesized on Rink Amide MBHA resin (600 mg, loading: 0.7 mmol/g) using Fmoc–Gly–OH, Fmoc–Cys(Trt)–OH, Fmoc–Asp(OcHex)–OH and Fmoc–Arg(Tos)–OH. The product was cleaved using TFA cleavage (94% TFA, 2.5% H_2O, 2.5 EDT, 1.0% TIS) for 2 h at room temperature to give 340 mg of **1**.

Disulfide bridge formation. Intermediate **1** (310 mg, 0.367 mmol) was dissolved in TFA/anisole (19 : 1, 310 ml), chilled and cyclized upon addition of $Tl(tfa)_3$ (200 mg, 0.367 mmol 1.0 equiv.). After stirring at 4 °C for 1 h, the reaction mixture was concentrated by evaporating the solvent under reduced pressure; the peptide was then precipitated with diethyl ether (35 ml) and centrifuged. Trituration with diethyl ether/centrifugation cycle was repeated three more times to ensure that the thallium salts were removed. The precipitate was dissolved in 1% aq HOAc (10 ml) and lyophilized to give 290 mg of the cyclic peptide **2**. Portion of the product was dissolved in dioxane and 1.1 equiv. of HCl (6 M solution in dioxane) was added and solvent was removed under reduced pressure to give **2** as hydrocloride, which was used for coupling reactions.

Synthesis of Octapeptide 4

Ac-*D*-Phe–Pro–Arg(Tos)–Pro–Gly–Gly–Gly–Gly–OH **4** was synthesized on Wang resin (450 mg, loading: 0.9 mmol/g) using Fmoc–Gly–OH, Fmoc–Pro–OH, Fmoc–Arg(Tos)–OH and Ac-*D*-Phe–OH. First amino acid (Fmoc–Gly–OH) was coupled using symmetrical anhydride method [15], and level of the first residue attachment was determined to be 83%. The product was cleaved using TFA cleavage (95% TFA, 2.5% H_2O, 2.5% TIS) for 2 h at room temperature. Solvent was evaporated, then diethyl ether was added and the precipitate was filtered off and washed with diethyl ether. A crude product (290 mg) was purified using FPLC (gradient: 15% B → 60% B in 45 min, $r_t = 53$ min).

Synthesis of Peptide Conjugate 6

Amino component **2** (108 mg, 0.115 mmol) was dissolved in 5 ml of DMF, carboxyl component **4** (84 mg, 0.10 mmol) and HOBt (15 mg, 0.12 mmol) were added. After cooling the stirred mixture to −10 °C, WSC (26 μl, 23 mg, 0.12 mmol) was added and the mixture was allowed to warm to room temperature.

DOI: 10.1002/psc

Table 1 MS, HRMS and HPLC data of the synthesized compounds

Compounds	Compositon	MS (Calculated)	MS found	t_r HPLC (min)	Purity (%)
1	$C_{33}H_{52}N_{10}O_{10}S_3$	844.30	845.3 ($[M+H]^+$)	13′ 58″[a]	100
2	$C_{33}H_{50}N_{10}O_{10}S_3$	842.29	843.3 ($[M+H]^+$)	13′ 59″[a]	100
3	$C_{20}H_{34}N_{10}O_8S_2$	606.20	607.2 ($[M+H]^+$)	4′ 07″[a]	100
4	$C_{42}H_{57}N_{11}O_{12}S$	939.39	940.4 ($[M+H]^+$)	14′ 05″[a]	100
5	$C_{35}H_{51}N_{11}O_{10}$	785.38	786.4 ($[M+H]^+$)	12′ 31″[a]	100
6	$C_{75}H_{105}N_{21}O_{21}S_4$	1763.67	883.3 ($[M+2H]^{2+}$)	39′ 38″[b]	100
7	$C_{55}H_{83}N_{21}O_{17}S_2$	1373.57	688.0 ($[M+2H]^{2+}$)	21′ 16″[b]	100
8	$C_{19}H_{28}N_6O_6$	437.2149 ($[M+H]^+$)	437.2138[d] ($[M+H]^+$)	10′ 50″[c]	100
9	$C_{22}H_{32}N_6O_6$	477.2462 ($[M+H]^+$)	477.2450[d] ($[M+H]^+$)	11′ 37″[c]	100
10b	$C_{21}H_{32}N_8O_6$	493.2523 ($[M+H]^+$)	493.2532[d] ($[M+H]^+$)	7′ 49″[c]	93.2
15	$C_{18}H_{28}N_6O_4$	393.2250 ($[M+H]^+$)	393.2234[d] ($[M+H]^+$)	9′ 38″[c]	98.2
16	$C_{21}H_{24}N_4O_4$	397.1876 ($[M+H]^+$)	397.1871[d] ($[M+H]^+$)	12′ 02″[c]	95.9
17	$C_{24}H_{22}N_4O_4$	431.1719 ($[M+H]^+$)	431.1733[d] ($[M+H]^+$)	14′ 44″[c]	97.4
18	$C_{24}H_{28}N_4O_5$	453.2138 ($[M+H]^+$)	453.2138[d] ($[M+H]^+$)	13′ 19″[c]	100

[a] Gradient: 1% B → 95% B in 27 min.
[b] Gradient: 1% B → 56% B in 40 min.
[c] Gradient: 10% B → 80% B in 30 min.
[d] HRMS.

After 4 h DMF was evaporated under reduced pressure, the crude product was triturated with 0.1 M $NaHCO_3$, filtered off and successively washed with ice-cold water. Product was purified using FPLC to give 52 mg (45%) of **6** (gradient: 15% B → 70% B in 110 min, $r_t = 79$ min).

HF Deprotection of 2, 4 and 6

The protected peptides **2**, **4** and **6** (50 mg each) were treated with liquid HF in the presence of *p*-cresol (1 ml) as scavenger [16] for 2 h at 0 °C, then diethyl ether was added, the precipitate was filtered off and washed with diethyl ether to give fully deprotected peptides **3**, **5** and **7**, respectively.

Synthesis of Tripeptides 8 and 9

First amino acid (Fmoc–Asp–OBzl) (94 mg, 0.21 mmol) was coupled via β-carboxylic group on 2-chlorotrityl chloride resin (150 mg, 1.4 mmol/g) in the presence of DIEA (87 µl, 68 mg, 0.53 mmol) in dichloromethane for 1 h. Methanol (300 µl) was added and the slurry was left aside for 10 min, and level of the first residue attachment was determined to be 63%. Resin was split to two equal portions whereupon Fmoc–Pro–OH or Fmoc–Gly–OH in the first step and Fmoc–Arg(Pbf)–OH in the last step were coupled. Product was cleaved from resin using acetic acid cleavage (AcOH : H_2O : $CF_3CH_2OH = 8:1:1$) for 30 min at room temperature. Solvents were removed under reduced pressure. Pbf protecting group was cleaved with TFA cleavage (95% TFA, 2.5% H_2O, 2.5% TIS) for 1 h at room temperature. Solvents were removed under reduced pressure, then diethyl ether was added, the precipitate was filtered off and washed with diethyl ether. Crude products were purified using semipreparative HPLC.

Synthesis of *N*-benzylated Tetrapeptide 10b

Peptide **10b** was synthesized on Rink amide MBHA resin (120 mg, loading: 0.9 mmol/g). Fmoc–Gly–OH and Fmoc–Asp(O*t*Bu)–OH were coupled sequentially to the resin. After Fmoc group removal, DMF was added in minimal amount to swell the resin with bound Asp(O*t*Bu)–Gly–NH_2, then benzaldehyde (10 equiv.) was added and the reaction was allowed to proceed for 2 h at room temperature. Resin was washed three times with DMF and again benzaldehyde (10 equiv.) was added. After 2 h the resin was washed five times with DMF, 5 equiv. of $NaBH_3CN$ were added and the mixture was left overnight at room temperature. Resin was washed five times with DMF, swollen in minimal amount of DMF, Fmoc–Gly–Cl (5 equiv.) dissolved in the minimal amount of pyridine was added and allowed to react for 4 h at room temperature. The last cycle was repeated once more, whereupon Fmoc–Arg(Pbf)–OH was coupled as the fourth amino acid. The product was cleaved (95% TFA, 2.5% H_2O, 2.5% TIS) for 2 h at room temperature. Solvents were removed under reduced pressure, then diethyl ether was added, the precipitate was filtered off and washed with diethyl ether giving **10b** (26 mg) which was purified using semipreparative HPLC.

Synthesis of *N*-benzylated Peptide 15 and Peptidomimetics 16, 17 and 18

Wang resin (450 mg, loading: 0.9 mmol/g) was swollen in minimal amount of DMF, 5 equiv. of Fmoc-β-Ala–Cl (for preparation of **15** and **16**) or Fmoc–Gly–Cl (for preparation of **17** and **18**) dissolved in minimal amount of pyridine was added and coupling was allowed to proceed for 4 h at room temperature. Fmoc protecting group was removed, minimal amount of DMF was added to swell the resin, then 10 equiv. of benzaldehyde was added and after 2 h the resin was washed three times with DMF. Addition of benzaldehyde and washing was repeated once more, 5 equiv. of $NaBH_3CN$ was added and left overnight at room temperature, whereupon the resin was washed five times with DMF and swollen in minimal amount of DMF. Fmoc–Gly–Cl (5 equiv.) (for **15**), Fmoc-β-Ala–Cl (5 equiv.) (for **16**), Fmoc–PABA–Cl (5 equiv.)

DOI: 10.1002/psc

(for **17**) or Fmoc–MABA-Cl (5 equiv.) (for **18**) dissolved in a minimal amount of pyridine was added and coupling was allowed to proceed for 4 h. This cycle was repeated once more. After deprotection 4-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)benzoic acid [17] (for **16** and **17**), Fmoc–Arg(Pbf)–OH (for **15**) or Fmoc–Gly–OH and Fmoc–Isn–OH (for **18**) was coupled to the resin using DIC/HOBt method. Peptidomimetics were cleaved from the resin (95% TFA, 2.5% H_2O, 2.5% TIS) for 2 h at room temperature and purified using semipreparative HPLC to afford **15** and **18**. After removal of benzamidine-protecting group by catalytic hydrogenation (H_2, Pd/C, EtOH, 1 bar, overnight) **16** and **17** were obtained.

Docking Studies

Autodock 3.05 [18] was used to predict ligand-binding mode in the thrombin active site and fibrinogen receptor binding site. For the compounds **8, 9, 10b, 15, 16, 17** and **18** most favorable energetic conformation was found with Hyperchem using semi-empirical method MNDO [19]. All amide bonds had *trans* configuration and were marked as nonrotatable. Compounds were protonated on the basic center (guanidine, amidino or amino group) and were assigned negative charge on acidic center (β carboxylic group of aspartate or its bioisoster). For docking calculations we used crystal structure of thrombin with resolution of 2.4 Å (PDB: **1KTS**) [20] and crystal structure of $\alpha_{IIb}\beta_3$ receptor with resolution of 2.9 Å (PDB: **1TY5**) [21]. Number of runs was 250. Other parameters were set as default. For the interpretation of docking results the ligand with the lowest docked energy was chosen.

Biological Assays

Enzyme assay for determination of inhibition of thrombin. The ability of the synthesized compounds to inhibit the enzymatic activity of thrombin was measured as described previously [22] by amidolytic enzyme assay using chromogenic substrates and is expressed as inhibition constant K_i. Values for K_i were calculated according to Cheng and Prusoff [23] based on a relation between reaction velocity equations in the absence and presence of inhibitor, using the relevant K_m [24]. Argatroban ($K_i = 6.23 \pm 1.59$ nM) was used as control.

Inhibition of in vitro binding of fibrinogen to isolated $\alpha_{IIb}\beta_3$ integrin. The binding affinities of the synthesized compounds to human integrin $\alpha_{IIb}\beta_3$ were measured by a solid-phase competitive displacement assay using biotinylated fibrinogen as ligand [22,25] as previously described.

Inhibition of in vitro human platelet aggregation in platelet-rich-plasma. Healthy male donors who have not taken any antiplatelet drugs for at least 2 weeks were made to fast for 8 h prior to drawing blood; then 10 ml whole blood was collected using a butterfly needle and 10 ml plastic syringe with 1 ml of 0.129 M buffered sodium citrate (3.8%). Platelet-rich plasma (PRP) was prepared by centrifugation at $1000 \times g$ for 10 min at room temperature, allowing the centrifuge to coast to a stop without braking. Platelet poor plasma (PPP) was prepared by centrifuging the remaining blood at $2000 \times g$ for 15 min at room temperature allowing the centrifuge to coast to a stop without braking. The PRP was adjusted with PPP to a count of $250 \pm 25 \times 10^6$ ml. To the cuvette, 50 µl of adenosine 5′diphosphate (ADP) (50 µM) was added and the aggregation was monitored for 10 min. The entire procedure was run within 2 h, since this is the maximal viability of the platelets. Saline instead of test compound was used to determine the maximal aggregation. Percentage of inhibition of aggregation at 50 µM concentration was calculated as follows:

$$\%\ \text{inhibition of aggregation} = \left(1 - \frac{\%\ \text{aggregation}_{\text{inhibitor}}}{\%\ \text{aggregation}_{\text{control}}}\right), \text{ where}$$

$$\%\ \text{aggregation} = \frac{OD_{\text{starting}} - OD_{\text{ending}}}{OD_{\text{starting}} - OD_{\text{PRP}}}$$

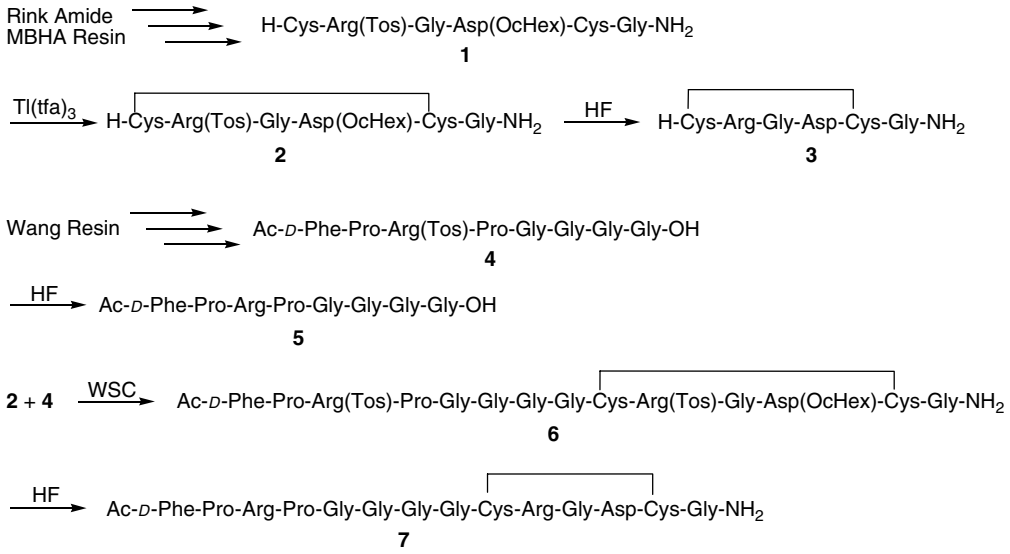
OD—optical density

RESULTS AND DISCUSSION

Chemistry

Our synthetic plan was to synthesize protected peptides **2** and **4** on solid phase and then couple them in solution to give peptide conjugate **6**, which after deprotection would afford the conjugate **7**. We combined synthesis on solid phase and in solution in order to get access to peptides **3** and **5** also, required as control compounds in biological testing, by deprotection of **2** and **4** (Scheme 1). Rink amide MBHA resin and Wang resin were chosen as solid supports for peptide synthesis enabling us to use TFA cleavage affording protected peptides, which were later fully deprotected using HF cleavage. We employed Fmoc protected amino acids with TFA-resistant side chain protecting groups, i.e. tosyl protection for arginine and cyclohexyl ester protection for aspartic acid. Cystein with TFA-resistant acetamidomethyl side chain protection was initially used in the synthesis of peptide **2** which was planned to be prepared in solution by simultaneous removal of the sulfhydryl protecting group and disulfide bond formation from Cys(Acm) analog of **1**. However, HPLC and MS analysis demonstrated that a substantial amount of aspartimide derivative ($m/z = 887.3$) of the desired product was formed. Therefore, cystein with trityl (Trt) side chain protection was instead employed and, using this strategy, after TFA cleavage peptide **1** was smoothly obtained. Several methods and reagents for disulfide bond formation in **1** were tried, including air oxidation in ammonium acetate buffer, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), iodine, ($K_3[Fe(CN)_6]$) and thallium(III) trifluoroacetate [16]. The latter one was found to be the most favorable, although not the most practical due to high toxicity of thallium compounds. In the synthesis of linear peptide **4**, besides the main product, HPLC showed two overlapping side peaks with +57 ($m/z = 997.4$) and +114 ($m/z = 1054.4$) higher molecular mass, indicating undesired insertion of one or two additional glycine residues. Since this problem occurred also in the second synthetic attempt, we concluded that reaction condition or impurity in reagents was responsible for formation of undesired

DOI: 10.1002/psc



Scheme 1 Synthesis of peptide conjugate **7**.

side products that were efficiently removed by FPLC purification.

Although solution condensation of peptides **2** and **4** using WSC/HOBt method was expected to be the most critical step, it proceeded fast and in good yield affording pure peptide conjugate **6** after FPLC purification.

Synthesis of tripeptides **8** and **9** was carried out on 2-chlorotrityl chloride resin. Owing to high reactivity of 2-chlorotrityl chloride resin, coupling of β carboxylic group of Fmoc–Asp–OBzl to the resin was successful using only 1 equiv. of Fmoc protected amino acid in the absence of activating agent (Figure 1).

The synthesis of tripeptide amide **10a** was attempted on Rink Amide MBHA resin. In the first step Fmoc–Asp(O*t*Bu)–OH was coupled to the resin. Formation of imine with benzaldehyde and following sodium cyanoborohydride reduction is the efficient way to perform benzylation under mild conditions on solid support [26–28]. However, HPLC of the intermediate cleaved from the resin with TFA after the benzylation step showed several peaks which occurred probably due to instability of Bzl–Asp(O*t*Bu)–NH_2 attached to Rink amide MBHA resin. To confirm this, glycine was inserted between Rink amide resin and protected aspartic acid. HPLC and MS showed that with this modification the desired product Bzl–Asp–Gly–NH_2 was obtained in satisfying purity, so it was decided to proceed with the synthesis of **10b** instead of **10a** to avoid synthetic problems.

As expected, coupling to a secondary amino group of benzylated amino acid turned out to be very difficult in the synthesis of **10b** and **15**–**18** (Scheme 2). Neither mixed anhydride method nor employing efficient coupling reagents, e.g. HATU did not show substantial coupling. The use of Fmoc protected amino acid chlorides was found to be most suitable for the synthesis of compounds **15**, **16**, **17** and **18**.

Biological Activity

In peptide conjugate **7** thrombin inhibition lead compound D-Phe–Pro–Arg and fibrinogen receptor antagonistic sequence Arg–Gly–Asp were joined via a polyglycine linker in order to combine anticoagulant and antiplatelet aggregation activities in one molecule. In tripeptides **8** and **9** comprising guanidino and carboxylic acid moieties required for interaction with fibrinogen receptor, thrombin inhibitory activity was built in by introducing benzyl ester group which, according to molecular modeling, should fill thrombin S_3 pocket. In contrast to peptide conjugate **7**, low molecular weight tripeptides **8** and **9** possess highly integrated sequences fPR and RGD which would allow interaction with both targets. In compounds **10b** and **15**–**18** the aromatic ring to fill the thrombin S_3 pocket is provided by *N*-benzyl moiety. Additionally, in compounds **15**–**18** arginine and aspartic acid side chains bearing ionic groups, as well as glycine residue were bioisosterically replaced giving peptidomimetic compounds with potential dual antithrombotic activity.

Biological activities of the synthesized compounds are presented in Table 2. It is somehow disappointing that conjugate **7**, comprising both D-Phe–Pro–Arg and Arg–Gly–Asp pharmacophores, did not exhibit any substantial thrombin inhibitory effect. Comparison with thrombin inhibitory activity of linear peptide **5**, also containing D-Phe–Pro–Arg sequence shows that absence of thrombin inhibition originates from inactivity of **5**. The conjugate **7** was designed by linking thrombin active site inhibitor D-Phe–Pro–Arg via a polyglycine linker with antiaggregarory RGD sequence in order to combine anticoagulant and antiplatelet aggregation activities in one molecule. We incorporated proline after the D-Phe–Pro–Arg sequence to ensure that the cleavage of Arg–Pro

DOI: 10.1002/psc

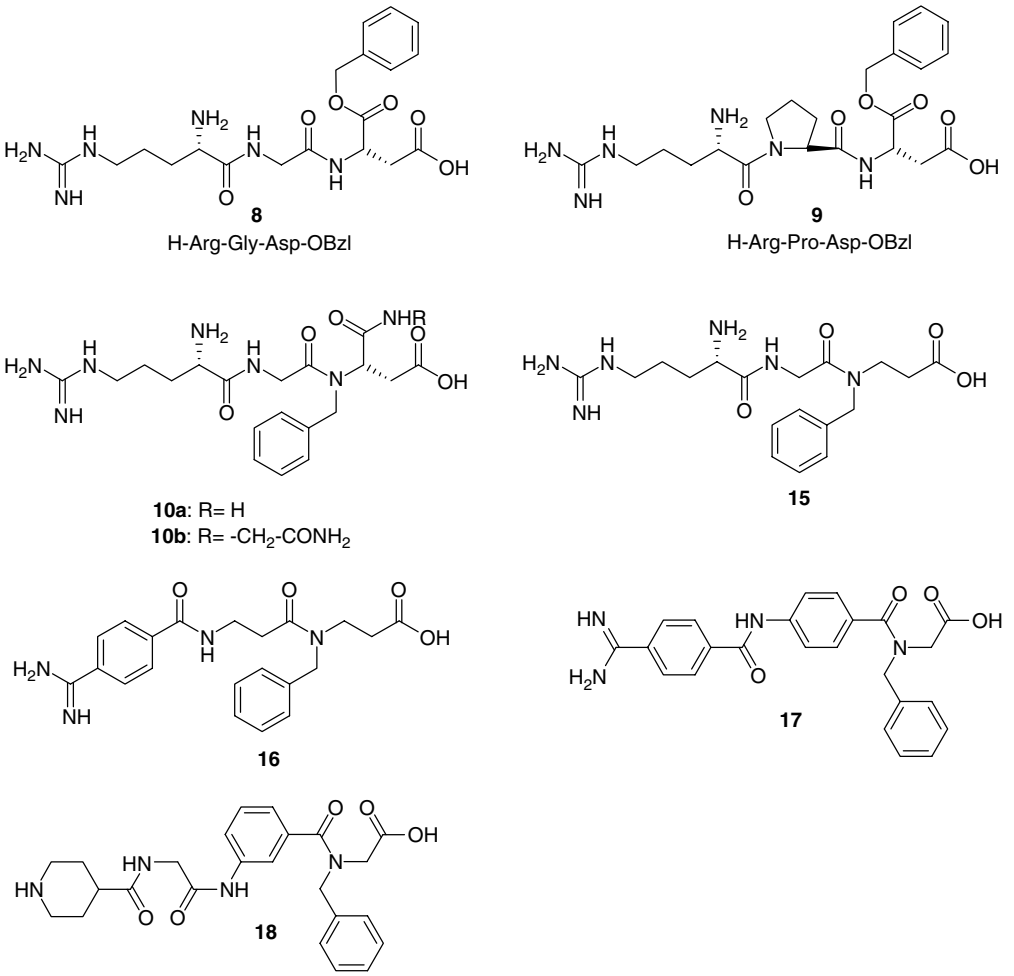
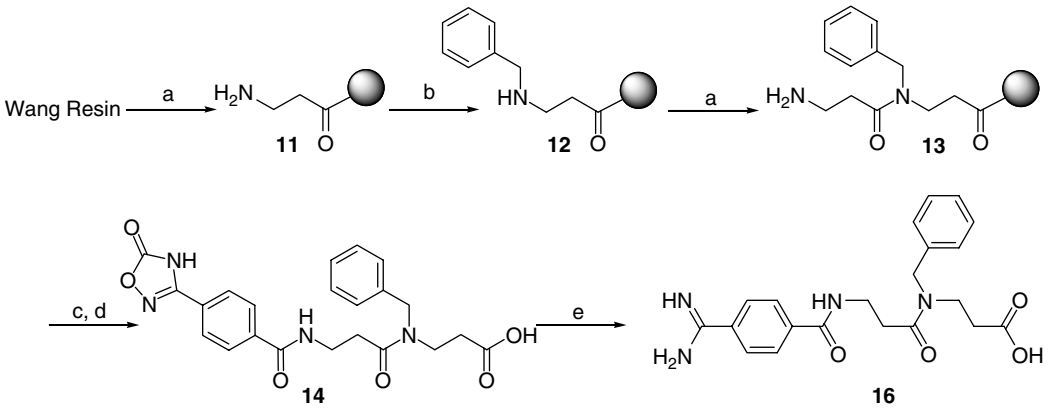


Figure 1 Structures of tripeptides **8** and **9**, pseudopeptides **10** and**15** and peptidomimetics **16**–**18**.



(a) Fmoc-ß-Ala-Cl, DMF/pyridine, rt, 2 h (b) benzaldehyde, $NaBH_3CN$, rt, overnight
(c) 4-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)benzoic acid, DIC, HOBt, DMF, 4 h
(d) 95 % TFA, 2.5 % H_2O, 2.5 % TIS, 2h (e) H_2, Pd/C, rt, overnight.

Scheme 2 Synthesis of peptidomimetic compound **16**.

peptide bond would not occur, as already exploited in the case of bivalirudin [29]. To avoid cleavage of Arg–Gly bond in RGD sequence by thrombin, cyclic peptide c(Cys–Arg–Gly–Asp–Cys) was chosen, as cyclic peptides are usually resistant to enzyme degradation. Tetraglycine linker had been shown previously to be useful in designing conjugates with good thrombin inhibitory activity [8]. A possible reason for low thrombin inhibitory potency of **5** and **7**, due to their special substitution pattern may reside in unexpected

DOI: 10.1002/psc

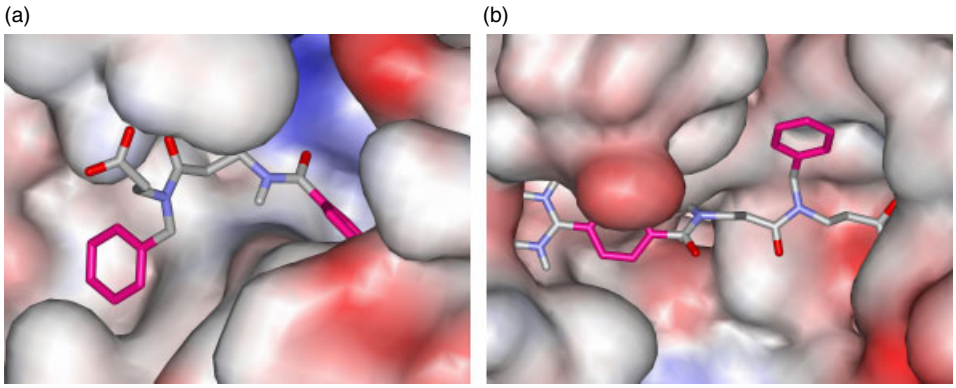


Figure 2 Compound **16** docked into the active site of thrombin (a) and to the fibrinogen receptor-binding site (b).

Table 2 Biological activities of synthesized compounds

Compounds	K_i (Thrb)	IC_{50} ($\alpha_{IIb}\beta_3$)	% inhibition (PRP)[a]
3	>100 μM	1.9 μM	62.5%
5	>100 μM	nd	nd
7	>100 μM	3 μM	54.3%
8	200 μM	3 μM	38.9%
9	65 μM	110 μM	0%
10b	>100 μM	>100 μM	0%
15	>100 μM	8 μM	7.7%
16	>100 μM	0.3 μM	88.7%
17	>100 μM	7.8 μM	58.3%
18	>100 μM	>100 μM	0%

[a] % of inhibition of aggregation at 50 μM concentration.
nd, not determined.

cleavage of Arg–Pro bond between arginine and proline. This appears a bit controversial having in mind a successful story of bivalirudin; however, cleavage of Arg–Pro bond by thrombin has been demonstrated in the case of D-Phe–Pro–Arg–Pro–$(Gly)_4$ sequence [30]. The conjugate **7** and its fragment peptide **3** both containing RGD sequence displayed comparable fibrinogen receptor antagonistic activity, showing that peptide **5** attached to **3** neither reduced nor enhanced the RGD antagonistic activity of **3**.

As expected, replacement of glycine in H–Arg–Gly–Asp–OBzl (**8**) with proline to give **9** increased thrombin inhibitory potency three-fold and reduced fibrinogen receptor antagonistic activity by a factor of 37. As suggested by docking experiments into the thrombin active site, in compound **10b** a benzyl moiety was introduced on aspartate amino group but unfortunately, this step had detrimental effect on both thrombin inhibition and fibrinogen receptor antagonistic activity. Replacement of Asp–Gly–NH_2 moiety in **10b** with β-alanine gave compound **15** which showed good fibrinogen receptor antagonistic activity. Compound **16** in which arginine of **15** was replaced with benzamidine moiety, a classical arginine surrogate [31], and Gly was replaced with β-Ala to retain the proper geometry of the molecule, displayed the best fibrinogen receptor antagonistic activity (IC_{50} = 0.3 μM) that was in good correlation with its anti-aggregatory activity (89% inhibition at 50 μM concentration). Figure 2 shows compound **16** docked into the active site of thrombin and to the fibrinogen receptor-binding site. However, neither **10b**, nor **15** and **16** had a measurable thrombin inhibitory activity. Compounds **10b, 15** and **16** possess Gly or β-Ala as the central amino acid residue, which are not bulky enough to have good interactions with thrombin S_2 binding pocket, occupied by proline in the D-Phe–Pro–Arg sequence, which results in poor thrombin inhibition. To overcome this, compounds **17** and **18** possessing *p*-aminobezoic acid and *m*-aminobenzoic acid as the central scaffold which could have interactions with the S_2 pocket of thrombin, were prepared. Additionally, in **17** and **18** the terminal β-Ala was replaced with Gly to tune the distance between the charged groups. Compound **17** showed fibrinogen receptor antagonistic activity comparable to that of **15** and 25-fold lower from that of less rigid **16**. The absence of thrombin inhibitory activity of **17** may be due to prevention of optimal positioning of benzamidine and *p*-aminobenzoic acid moieties by the rigid amide bond between both aromatic rings. Compound **18**, possessing isonipecotic acid as less basic arginine replacement and *m*-aminobenzoic as central scaffold did not show any substantial activity on both targets.

CONCLUSIONS

Lead peptides D-Phe–Pro–Arg for thrombin inhibition and Arg–Gly–Asp for fibrinogen receptor antagonism were incorporated in a peptide conjugate **7** which displayed fibrinogen receptor antagonistic activity but, unfortunately, did not inhibit thrombin. A tripeptide H–Arg–Pro–Asp–OBzl (**9**) which was designed and prepared as a multiple ligand comprising highly integrated pharmacophore groups of both D-Phe–Pro–Arg and Arg–Gly–Asp lead sequences displayed a moderate

DOI: 10.1002/psc

but well balanced thrombin inhibition and fibrinogen receptor antagonistic activity. A series of *N*-benzyl peptidomimetics **15**–**17** possessed good fibrinogen receptor antagonistic activity but were devoid of thrombin inhibitory activity, most probably due to lacking sterical interaction with the S_2 binding pocket of thrombin.

The results of this work demonstrated the possibility to combine peptide leads *D*-Phe–Pro–Arg for thrombin inhibition and Arg–Gly–Asp for antagonistic activity on fibrinogen receptor or their mimetics in one molecule acting both as thrombin inhibitor and as fibrinogen receptor antagonist. However, further optimization is required to achieve optimal merging of both pharmacophores and to increase potency on both targets.

Acknowledgements

This work was financially supported by Slovenian Research Agency (Grant No. P1-0208) and by the Hungarian Ministry of Education (Medichem 2 1/A/005/2004). The authors thank Professor Mojca Stegnar for enabling us to perform biological tests in her laboratory, Professor Roger Pain for critical reading of the manuscript and Dr Marko Anderluh for kindly donating of 4-(5-oxo-4,5-dihydro-1,2,4-oxadiazol-3-yl)benzoic acid.

REFERENCES

1. Lopez AD, Mathers CD, Ezzati M, Jamison DT, Murray CJL. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. *Lancet* 2006; **367**: 1747–1757.
2. Rang HP, Dale MM, Ritter JM, Moore PK. *Pharmacology*, 5th Edn. Churchill Livingstone: Edinburgh, 2003.
3. Kikumoto R, Tamao Y, Tezuka T, Tonomura S, Hara H, Ninomiya K, Hijikata A, Okamoto S. Selective inhibition of thrombin by (2*R*,4*R*)-4-methyl-1-[N^2-[1,2,3,4-tetrahydro-8-quinolinyl)sulfonyl]-L-arginyl] -2-piperidinecarboxylic acid. *Biochemistry* 1984; **23**: 85–90.
4. Schwienhorst A. Direct thrombin inhibitors – a survey of recent developments. *Cell Mol. Life Sci.* 2006; **63**: 2773–2791.
5. Sabatine MS, Tu TM, Jang IK. Combination of a direct thrombin inhibitor and a platelet glycoprotein IIb/IIIa blocking peptide facilitates and maintaines reperfusion of platelet-rich thrombus with alteplase. *J. Thromb. Thrombolysis* 2000; **10**: 189–196.
6. Wallenga JM, Jeske WP, Wallis DE, Bakhos M, Lewis BE, Leya F, Fareed J. Clinical experience with combined treatment of thrombin inhibitors and GP IIb/IIIa inhibitors in patients with HIT. *Semin. Thromb. Haemostasis* 1999; **25**(Suppl. 1): 77–81.
7. Marhardt F. Hirudin as an inhibitor of thrombin. *Methods Enzymol.* 1970; **19**: 924–932.
8. Maraganore JM, Bourdon P, Jablonski J, Ramachandran KL, Fenton JW II. Design and characterization of hirulogs: A Novel class of bivalent peptide inhibitors of thrombin. *Biochemistry* 1990; **29**: 7095–7101.
9. Knapp A, Degenhardt T, Dodt J. Hirudisins. Hirudin-derived thrombin inhibitors with disintegrin activity. *J. Biol. Chem.* 1992; **267**: 24230–24234.
10. Church FC, Phillips JE, Woods JL. Chimeric antithrombin peptide-characterization of an Arg-Gly-Asp (RGD)-containing and hirudin carboxyl terminus-containing synthetic peptide. *J. Biol. Chem.* 1991; **266**: 11975–11979.
11. Owen TJ, Broersma RJ, Krstenansky JL. Trifunctional antithrombin and antiplatelet peptides, WO9429349, 1994.
12. Morphy R, Kay C, Rankovic Z. Designed multiple ligands. An emerging drug discovery paradigm. *J. Med. Chem.* 2005; **48**: 6523–6543.
13. Beyermann M, Bienert M, Niedrich H, Carpino LA, Sadat-Aalaee D. Rapid continuous peptide synthesis via Fmoc amino acid chloride coupling and 4-(aminomethyl)piperidine deblocking. *J. Org. Chem.* 1990; **55**: 721–728.
14. Kaiser E, Colescott RL, Bossinger CD, Cook PI. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* 1970; **34**: 595–598.
15. Novabiochem Synthesis Notes, Attachment to hydroxymethyl resins using symmetrical anhydride, Catalog 2004/2005; 2.36.
16. Chan WC, White PD. *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*. Oxford University Press: New York, 2000.
17. Anderluh M, Cesar J, Štefanič P, Kikelj D, Janeš D, Murn J, Nadrah K, Tominc M, Addicks E, Giannis A, Stegnar M, Sollner Dolenc M. Design and synthesis of novel platelet fibrinogen receptor antagonists with 2*H*-1,4-benzoxazine-3(4*H*)-one scaffold. A systematic study. *Eur. J. Med. Chem.* 2005; **40**: 25–49.
18. Morris GM, Goodsell DS, Halliday RS, Huey R, Har TWE, Belew RK, Olson AJ. Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J. Comput. Chem.* 1998; **19**: 1639–1662.
19. HyperChem (Release 7.01 for Windows, Hypercube, Inc. 2002.
20. Hauel NH, Nar H, Priepke H, Ries U, Stassen J-M, Wienen W. Structure-based design of novel potent nonpeptide thrombin inhibitors. *J. Med. Chem.* 2002; **45**: 1757–1766.
21. Xiao T, Takagi J, Coller BS, Wang J-H, Springer TA. Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics. *Nature* 2004; **432**: 59–67.
22. Anderluh Štefanič P, Anderluh M, Ilaš J, Mravljak J, Sollner Dolenc M, Stegnar M, Kikelj D. Toward a novel class of antithrombotic compounds with dual function. Discovery of 1,4-benzoxazin-3(4*H*)-one derivatives possessing thrombin inhibitory and fibrinogen receptor antagonistic activities. *J. Med. Chem.* 2005; **48**: 3110–3113.
23. Cheng Y, Prusoff WH. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* 1973; **22**: 3099–3108.
24. Brandt RB, Laux JE, Yates SW. Calculation of inhibitor K_i and inhibitor type from the concentration of inhibitor for 50-percent inhibition for Michaelis-Menten enzymes. *Biochem. Med. Metab. Biol.* 1987; **37**: 344–349.
25. Addicks E, Mazitschek R, Giannis A. Synthesis and biological investigation of novel tricyclic benzodiazepinedione-based RGD analogue. *Chem BioChem* 2002; **3**: 1078–1088.
26. Miranda LP, Meutermans WDF, Smythe ML, Alewood PF. An activated $O \rightarrow N$ acyl transfer auxiliary: efficient amide-backbone substitution of hindered "difficult" peptides. *J. Org. Chem.* 2000; **65**: 5460–5468.
27. Nefzi AM, Ostresh JM, Houghten RA. Solid phase synthesis of 1,3,4,7-tetrasubstituted perhydro-1,4-diazepine-2,5-diones. *Tetrahedron Lett.* 1997; **38**: 4943–4946.
28. Akritopoulou-Zanze I, Sowin TJ. Solid-phase synthesis of macrolide analogues. *J. Comb. Chem.* 2001; **3**: 301–311.
29. Agnelli G. Clinical potential of oral direct thrombin inhibitors in the prevention and treatment of venous thromboembolism. *Drugs* 2004; **64**(Suppl. 1): 47–52.
30. Priestle JP, Rahuel J, Rink HM, Tones M, Grutter MG. Changes in interactions in complexes of hirudin derivatives and human α-thrombin due to different crystal forms. *Protein Sci.* 1993; **2**: 1630–1642.
31. Peterlin-Mašič L, Kikelj D. Arginine mimetics. *Tetrahedron* 2001; **57**: 7073–7105.

DOI: 10.1002/psc