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Original article

Synthesis and evaluation of a small library of graftable thrombin inhibitors derived from L-arginine

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Abstract

Novel piperazinyl-amide derivatives of *N*-α-(aryl-sulfonyl)-L-arginine were synthesized as graftable thrombin inhibitors, in the context of biomaterials' design. The possible disturbance of biological activity due to a variable spacer-arm fixed on the N-4 piperazinyl position and the introduction of a trifluoromethyl group as XPS (X-ray Photoelectron Spectroscopy) tag on the sulfonamide moiety were evaluated in vitro against human α-thrombin. All the compounds of the library were found to be active at the micromolar level, as the reference TAME (*N*-tosyl-L-arginine methyl ester). The blood compatibilization improvement of poly(ethylene terephthalate) (PET) membrane, coated or grafted by wet chemistry treatment with one representative inhibitor of the library, was also evaluated, showing interesting decrease in blood clot formation.

Keywords: Thrombin inhibitors; Arginine derivatives; Graftable inhibitors; Blood-compatible materials

1. Introduction

Thrombin is a key-enzyme in the blood coagulation system and represents a main target in medicinal chemistry [1]. Indeed, this enzyme is the final common mediator of both the intrinsic and extrinsic coagulation pathways; it triggers platelet activation, production of factors V, VIII, IX, and mediates the proteolytic cleavage of fibrinogen to fibrin [2]. Thrombin is formed by an A chain of 36 amino acids and a B chain of 259 amino acids connected by a disulfur bridge. This enzyme contains the catalytic triad (Asp-102, His-57, Ser-195) characteristic of the chymotrypsin family. From X-ray diffraction data, three important binding site pockets have been identified: the specificity pocket S1 with Asp-189, the hydrophobic proximal pocket S2 (also called P-pocket) and a larger hydrophobic distal pocket S3 (also called D-pocket) [3,4].

Many strategies for preventing and treating thromboembolic events have focused on the inhibition of thrombin generation, as well as on the inhibition of the thrombin action [5]. Heparin was in clinical use for more than 50 years. This natural compound (linear polysaccharide with alternating uronic acid and glucosamino units bearing carboxyl, sulfonic acid and sulfonamide groups) acts as an anticoagulant by activating antithrombin, which then inhibits thrombin, but its therapeutic use suffer severe limitations [6]. The quest for the ideal anticoagulant has produced several synthetic agents (molecules of low molecular weight) acting as direct thrombin inhibitors [1]. The covalently bound inhibitors (binding to Ser-195) are electrophilic compounds derived from the tripeptide (D)-Phe-Pro-Arg (fibrinogen recognition sequence), with the peptide arginal Efegatran and the chloromethylene ketone PPACK as leads [7]. High affinity inhibitors (non-covalently bound, interacting with Asp-189), called steric inhibitors, were also designed to block thrombin activity. In this family, the lead compounds are *N*-tosyl-(L)-arginine methyl ester (TAME) and benzamidine. Argatroban [8] and Ximelagatran [9] belong

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to this class of reversible thrombin inhibitors; they are presently the only synthetic inhibitors approved and available for medical purpose.

In our laboratory, we use active molecules stemming from medicinal chemistry approaches as tools for the biocompatibilization of polymer materials. Our strategy relies upon the covalent grafting of biologically active molecules, via a spacer-arm, on the surface of polymer devices. In this way, we confer specific properties selectively to the material surface, while the physico-chemical and mechanical properties of the bulk remain unchanged [10]. This methodology has been successfully applied to the preparation of novel supports for the in vitro mammalian cells' cultivation: the grafting of peptidomimetic molecules of the Arg-Gly-Asp (RGD) sequence, designed to interact with $\alpha_V\beta_3$ integrin receptor, promoted Caco-2 cell adhesion on poly(ethylene terephthalate) track-etched membranes [11].

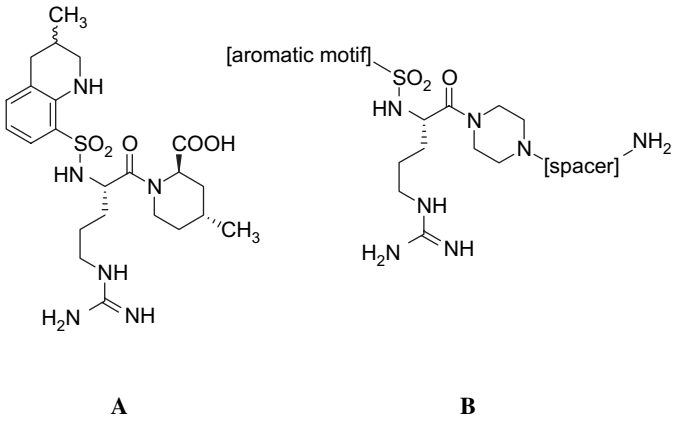
Now we are interested in blood-compatible materials. Polymer devices preventing clot formation are generally heparinized materials. Heparin can be attached to blood-contacting devices according to material surface available functional groups and suitable treatments: activation of heparin carboxylic groups with carbodiimides [12,13] or deaminative cleavage [14] and grafting on aminated surfaces; activation of surface amino groups with isocyanates [15] or glutaraldehyde [16–18]. Heparin-like materials were also produced by surface chemical modification. For example, unsaturated polymers were modified with *N*-chlorosulfonyl isocyanate [19–22]; isoprene with fuming sulfuric acid [23]. Sulfonated forms of polystyrene [24], dextran [25], chitosans [26] and more recently polyrotaxanes [27] were also prepared.

Thrombomodulin, an anticoagulant transmembrane protein found on endothelial cells' surface [28], was also investigated for the incorporation on biomaterials' surface in order to obtain non-thrombogenic devices [29–34]. Recently, nitric oxide releasing/generating substances were incorporated into polymer materials in order to avoid thrombosis and stenosis on biomedical implants [35–37]. Nitric oxide is in fact a well-known potent anti-platelet agent, preventing adhesion and activation of platelets, and a smooth cell proliferation inhibitor [38].

Instead, little attention has been devoted, so far, to the use of synthetic drugs for preventing blood coagulation on materials. We found only two representative examples: (i) an acrylamide derivative of Argatroban was graft-polymerized on the surface of polyurethanes [39], and (ii) a *p*-amino-benzamidine derivative was immobilized on maleic anhydride copolymer films via a spacer [40].

Our objective was to prepare hemocompatible polymer membranes by the surface grafting of thrombin inhibitors. In this context of biomaterials, the surface bound inhibitors are not allowed to be processed by the enzyme. We thus selected a simplified structure (**B**) of Argatroban (**A**) (steric inhibitor), equipped with a spacer-arm, as starting point of our research (Fig. 1).

Molecules **B** were derived from L-arginine and feature the following characteristics: (i) the guanidyl function for ionic interaction with Asp-189 in the S1-pocket; (ii) the lipophilic sulfonamide moiety for interaction with the S2–S3 binding sites;



A **B**

Fig. 1. Structure of the model compound Argatroban (A) and proposed structures of graftable thrombin inhibitors (B).

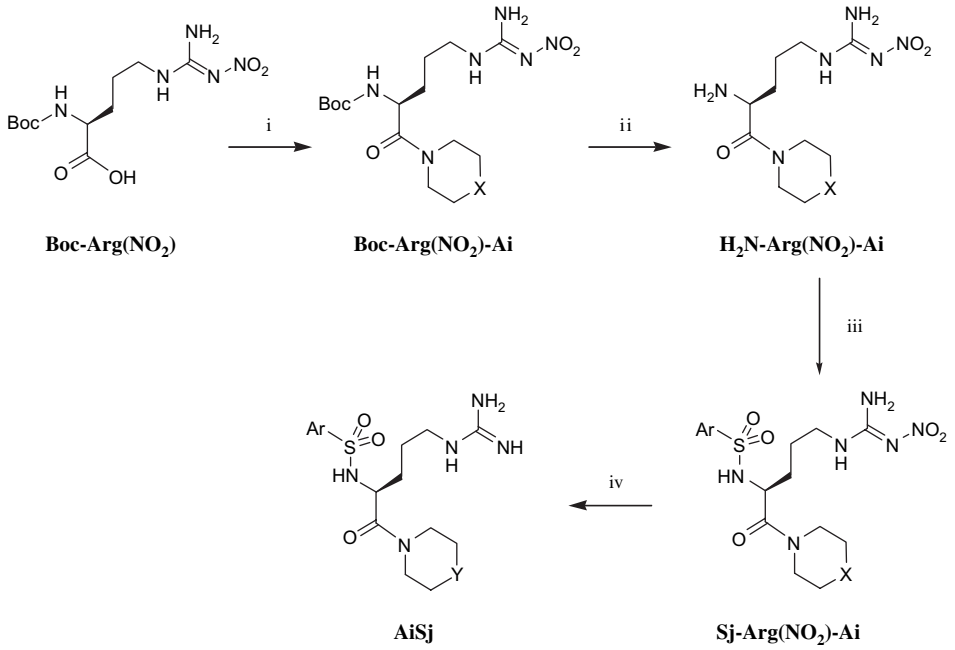
(iii) the piperazinyl amide moiety bearing a spacer-arm for surface grafting. Comparatively to Argatroban **A**, we suppressed the chiral centres on both the *N*- and *C*-substituents of the arginine template, and replaced the piperidine ring by a piperazine ring with the objective of using the N-4 atom as anchoring point of various spacer-arms. The validity of our design had to be established (theoretically and experimentally) before to use molecules **B** in any biomaterial application. A molecular modelling and docking study of the possible positioning of one representative of target molecules **B** (aromatic motif = *m*-trifluoromethyl-phenyl; spacer = propyl) into the thrombin active site, showed two modes of binding of similar energies, one of them allowing the spacer to point towards the surface of the enzyme and come out from the cavity [41].

In this article, we describe the synthesis of a small library of graftable thrombin inhibitors (**B**) and the evaluation of their activity against human thrombin in view to select good candidates for materials' hemocompatibilization.

2. Results and discussion

Compounds **B** (Fig. 1) were readily accessible according to Scheme 1: *N*,*N*′-protected arginine (Boc-Arg(NO_2)) was transformed into mixed anhydride and then coupled with an azacyclohexane derivative to furnish an amide (Boc-Arg(NO_2)-Ai, step i). After selective deprotection of the Boc group (H_2N-Arg(NO_2)-Ai, step ii), the arylsulfonyl motif was introduced by reaction with a chlorosulfonyl derivative (Sj-Arg(NO_2)-Ai, step iii). The final step was the deprotection of all masked functions (*N*-nitro-Arg and chain substituent X on the piperazine motif) by catalytic hydrogenation (AiSj, step iv) [42–44].

The experimental conditions of this four-step synthesis have been manually set up and exemplified with a few representatives (Aryl = *m*-trifluoromethyl-phenyl; Y = CH_2, NH, N-$(CH_2)_3$-NH_2). However, the optimization of our design of graftable inhibitors required the (parallel) synthesis of another series of compounds in order to address the following questions, when comparing Argatroban (**A**) and the



Scheme 1. Synthetic sequence adapted for parallel synthesis. Reagents and conditions: (i) NMM, $ClCO_2$-^{t}Bu (IBC), THF then azacyclohexane $HN(C_2H_4)_2X$, and NMM; (ii) TFA/CH_2Cl_2 (1:1), then aq. NaOH; (iii) ArylSO_2Cl, Et_3N, CH_2Cl_2; (iv) H_2, Pd/C, EtOH, HOAc; Y = X with deprotected terminal amino group.

target molecules (**B**): what could be the effect of (a) the replacement of the piperidine motif by a piperazine motif, (b) the presence of a spacer-arm on this motif, (c) the simplification of the aryl group structure, but with the introduction of a spectroscopic tag (CF_3) for XPS analysis of the biomaterial?

For this purpose, we selected nine building blocks for the synthesis of the carboxamide moiety (A1 to A9, Fig. 2) and

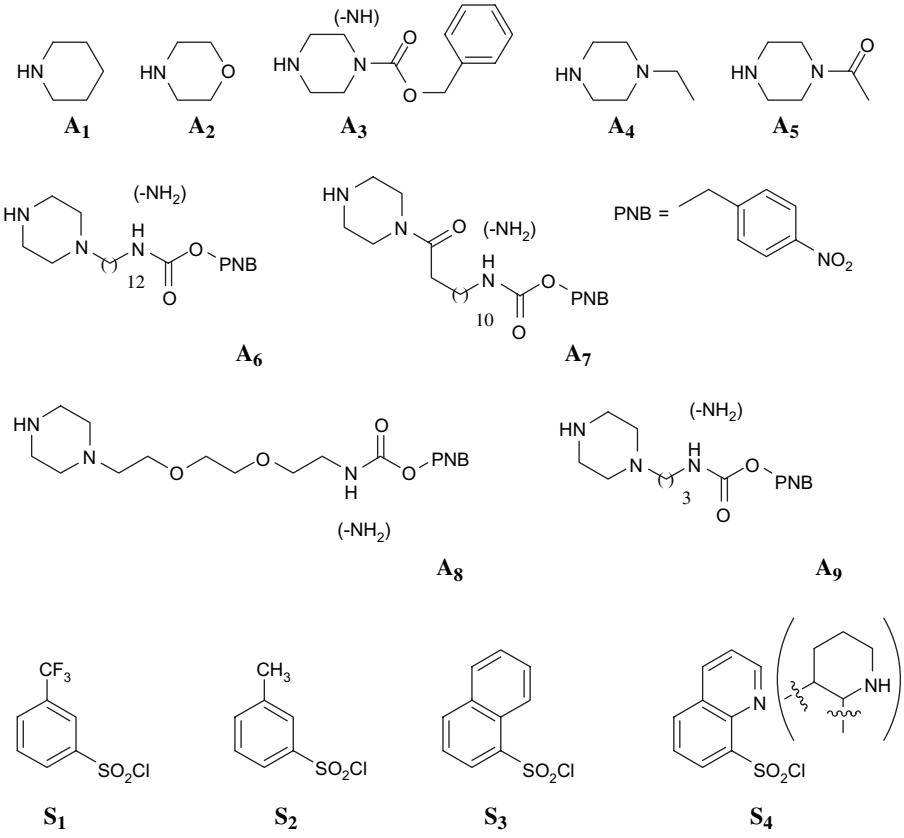


Fig. 2. Building blocks considered in parallel (and manual) synthesis. Residues after deprotection by hydrogenation are indicated in brackets.

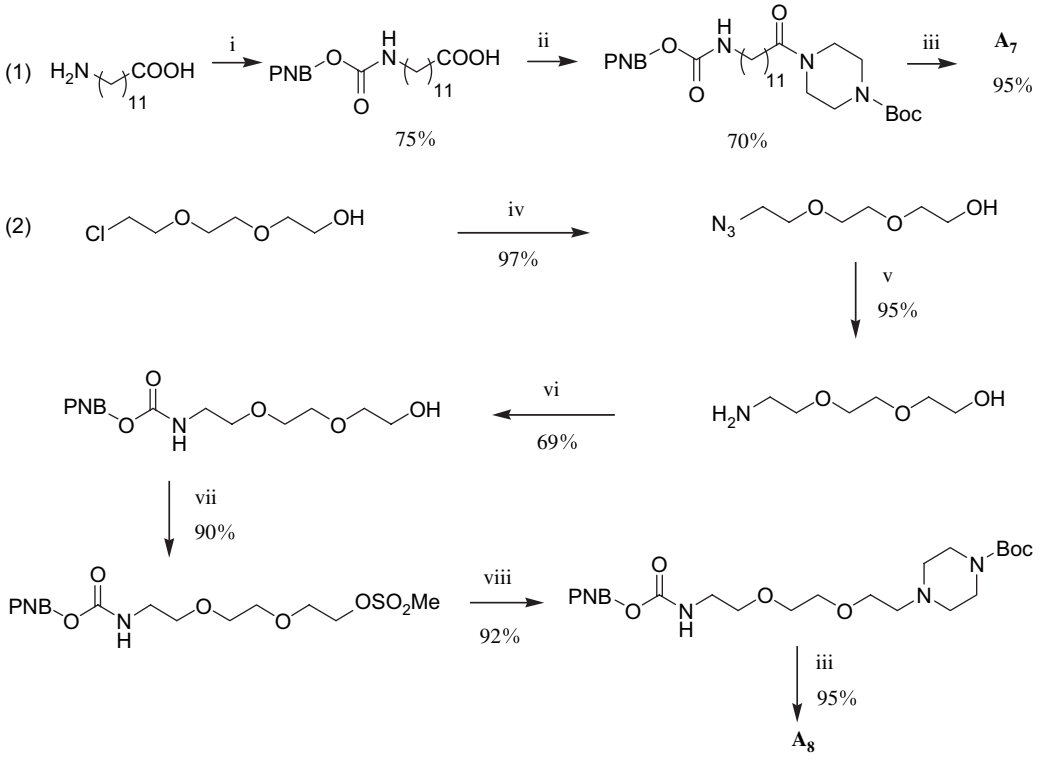
four building blocks for the synthesis of the sulfonamide moiety (S1 to S4, Fig. 2). S1 to S4, and A1 to A5 are commercially available, while A6 to A9 equipped with a spacer-arm have been prepared by conventional chemistry. A6 and A9 were previously described [41]. A7 was obtained in three steps from 12-amino-dodecanoic acid (Scheme 2, part 1): after *N*-protection with *p*-nitrobenzyl chloroformate, the carboxylic acid was coupled to 1-Boc-piperazine using the mixed anhydride method of activation; Boc deprotection with trifluoroacetic acid (TFA) and neutralization gave the block A7 in 50% overall yield. A8 resulted from the *N*-alkylation of 1-Boc-piperazine with 2-[2-[2-(*p*-nitro-benzyloxycarbonylamino]-ethoxy]-ethoxy]-ethyl mesylate followed by Boc cleavage (Scheme 2, part 2). The mesylate was obtained from 2-[2-[2-chloroethoxy]-ethoxy]-ethanol: after chloride substitution with azide, the azide was reduced by hydrogenation into amine which was protected as usual; the terminal alcohol was then activated by mesylation. The block A8 was recovered in 50% overall yield for six steps.

Our strategy for the introduction of a spacer-arm on the potential inhibitors **B** generates a novel basic centre, the N-4 atom of piperazine. The possible disturbance due to this function towards the enzyme recognition was evaluated, thanks to the blocks A1 to A5. The effect of the nature (hydrophobic or hydrophilic) and the length (3 to 12 atoms) of the spacer-arm was controlled with blocks A6 to A9; in all cases the terminal function for surface anchorage was a primary amine because the activated polymer surfaces usually display carboxylic or hydroxyl functions.

After surface grafting, the polymers were routinely analysed by X-ray photoelectron spectroscopy (XPS) for the quantification of fixed molecules. This requires a fluorine tag (CF_3) on the molecules of interest. The lipophilic sulfonamides moiety was used for that purpose (block S1); the effect of this structural modification on the biological activity was estimated thanks to blocks S2 to S4, S4 being close to Argatroban (Fig. 1, **A**).

The facilities of parallel automated synthesis (AutoChem from Mettler-Toledo) were used to prepare the library AiSj. (Scheme 1). The manual protocols used previously to synthesize A1S1, A3S1, A6S1 and A9S1 [41] had to be adapted: introduction of solutions of reagents instead of solids; reduction of volumes; suppression of concentration steps, when possible; suppression of intermediate chromatographic purifications; purifications by liquid–liquid extractions; NMR controls replaced by mass spectrometry (MS) and thin layer chromatography (TLC). Protocols are summarized in Table 1. Step i (C-functionalization of protected arginine; Scheme 1) was realized individually, with chromatographic purifications of Boc-Arg-(NO_2)-Ai, or in parallel synthesis, without other purifications than the liquid–liquid extractions.

After first deceiving results in performing step ii (Boc deprotection) with the automated system, due to pure TFA handling problems, we did not further investigate deprotection step in the parallel automated workstations and this operation was manually achieved. Step iii (*N*-functionalization with sulfonyl chlorides) used the crude TFA salts of amines [41] or preferably the free amines H_2N-Arg(NO_2)-Ai obtained by



Scheme 2. Synthesis of the piperazine linked spacer-arms. Reagents and conditions: (i) PNB-OCOCl, THF, NaOH·H_2O, 0 °C then H_3O^+; (ii) $ClCO_2$-^{t}Bu, Et_3N, THF, then 1-Boc-piperazine; (iii) TFA/CH_2Cl_2 (1:1), 20 °C then NaOH·H_2O; (iv) NaN_3, NaI, H_2O, 50 °C; (v) H_2, Pd/C, MeOH; (vi) PNB-OCOCl, Et_3N, CH_2Cl_2, 0 °C; (vii) $MeSO_2Cl$, pyridine, CH_2Cl_2, 0 °C; (viii) 1-Boc-piperazine, NaI, DIPEA, CH_3CN.

Table 1
Compared protocols

Step	Manual (optimized bench protocol)	Automated
i	Boc-Arg(NO_2) (1 equiv) and NMM (2 equiv) in THF (conc: 2%)	Boc-Arg(NO_2) (1 equiv) and NMM (2 equiv) in THF (conc: 5%)
	Addition of IBC (1 equiv) at −20 °C; 30 min at −20 °C	Addition of IBC (1 equiv) at 5 °C; 50 min at 5 °C
	Addition of azacyclohexane (1 equiv) and NMM (1 equiv) at −20 °C; 20 min at −20 °C; 1 h at RT	Addition of azacyclohexane (1 equiv) and NMM (1 equiv) at 5 °C; 50 min at 5 °C; 1 h at RT
	Concentration under vacuum (Rotavapor, 40 °C)	Filtration (TLC analysis); overnight evaporation under fume hood
	Extraction EtOAc/brine; drying over $MgSO_4$; evaporation	Extraction EtOAc/NaOH 1 N; evaporation of organic phase under fume hood
	Column chromatography on SiO_2	MS control of the crude
ii	Dissolution in TFA/CH_2Cl_2, 1:1 (conc: 3–5%); 2–3 h at RT	Addition of TFA/CH_2Cl_2, 1:1 (conc: 10%), 2 h at RT
	Concentration under vacuum (Rotavapor, RT)	Evaporation under fume hood
	Trituration in ether; solid washing with ether (three times)	Addition of ether; formation of a viscous oil
	Dissolution in NaOH 1 N and extraction with CH_2Cl_2 (or EtOAc)	
	Drying over $MgSO_4$; evaporation	
iii	(Free) amine (1 equiv) and Et_3N (2–4 equiv) in CH_2Cl_2 (conc: 3%)	TFA amine (1 equiv) and Et_3N (4–6 equiv) in CH_2Cl_2 (conc: 5–10%)
	Addition of sulfonyl chloride (1.2 equiv) at 0 °C	Addition of sulfonyl chloride (1.2 equiv), at 5 °C, in solution (S1 and S2 in CH_2Cl_2; S3 in CH_2Cl_2 + 2% DMF; S4 in pyridine)
	Stirring for 30 min at 0 °C and 2 h at RT	Shaking 1 h at 5 °C and 2 h at RT
	Washing with brine, drying over $MgSO_4$, concentration	Washing with brine
	Column chromatography on SiO_2	MS control of the crude. Purification by SFC
iv	Sulfonamide dissolved in EtOH/AcOH, 1:1 (conc: 5%)	Sulfonamide dissolved in EtOH/AcOH, 1:1 (conc 10%). MS control of the crude
	Pd 10% on C, H_2 (1 atm), 50 °C, 12 h	
	Filtration, concentration	
	Crystallization from H_2O/MeOH, 1:1 and washing with ether	

NMM = *N*-Methyl-morpholine; THF = tetrahydrofuran; IBC = isobutyl chloroformate; RT = room temperature; TFA = trifluoroacetic acid; MS = mass spectrometry; SFC = supercritical fluid chromatography; TLC = thin layer chromatography.

dissolution of the corresponding TFA salt in aqueous NaOH and extraction with CH_2Cl_2 or EtOAc [45,46] (manually or with the automate). According to the nature of the amine partner (TFA salt or free base), the amount of triethylamine used for the coupling with arylsulfonyl chlorides was adjusted (see Table 1). Similar yields were obtained following both protocols. These coupling reactions were readily performed in parallel synthesis. In all cases, the expected products Sj-Arg-(NO_2)-Ai were identified in the crude mixtures by MS and TLC, with two exceptions. In the A4 series, we recovered practically no materials after liquid–liquid extractions, and in the S4 series, the coupling products were identified in only two crudes (A5 and A8). Some products Sj-Arg-(NO_2)-Ai of the series A5, A6 and A8 have been purified by supercritical fluid chromatography (SFC). Finally, step iv (hydrogenation) was performed on chromatographed products (S1 series, manual synthesis) or on crude products (S2–S4 series, parallel synthesis) with similar results. The yields obtained from steps i to iv are collected in Table 2. Identifications of final products AiSj and intermediates by HRMS or elemental analysis are presented in Table 3.

The arginine derivatives AiSj were evaluated for their activity against human thrombin. The activities were measured by adapting the protocol of Lottenberg *et al*. [47]. A chromogenic substrate, H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride, was used as competitor. A mixture of substrate and tested compound at different concentrations was treated with the enzyme; the appearance of the substrate hydrolysis product (*p*-nitroaniline) was spectrophotometrically measured at 405 nm as a function of time. Increasing the inhibitor concentration resulted in a decrease of *p*-nitroaniline detection. The slope of the curve (absorbance versus time) gave the reaction rate and the velocity ratio V_0/V_i was calculated for each concentration of inhibitor (Graph 1). These values were plotted against the inhibitor concentration and the points were fitted with a linear curve (Graph 2). The inhibition constant K_i was calculated from the slope of the curve (m), knowing K_m and the substrate initial concentration [S] according to the equation:

$$K_i = \frac{K_m}{m} \frac{1}{([S] + K_m)}$$

The results are collected in Table 4. All the tested compounds were found to be active in the micromolar range, from 1 to 15 μM. In view to validate these rather low activities, a reference compound structurally close to the AiSj compounds and commercially available was tested. In our hands, the measured K_i of TAME (*N*-tosyl-L-arginine methyl ester) was 6–10 μM. The reported K_i of TAME [48] and Argatroban (structure A in Fig. 1) [49] are 40 μM and 20 nM, respectively. Thus, our compounds are definitively less active than Argatroban: the nanomolar activity of the drug has been lost with our structural modifications. Our biological results showed that the nature of the aryl group of the sulfonamide moiety has no great influence on the activity (compare series S1 to S2, S3, S4); hence, the *m*-trifluoromethyl-phenyl substituent remains a good choice, combining the requirements of thrombin inhibition and spectroscopic detection (XPS tag). On the other hand, the nature of the atom in position 4 of the azacyclohexane moiety (compare series A1, A2, A5), also poorly influenced the activity (Y = CH_2, O, N-Ac in Table 4). However, the presence of a basic motif (series A3; Y = NH) seemed

Table 2
Reaction yields

Blocks	Step			
	i	ii	iii	iv
A1	69	85		
S1			75/(>100)*	90
S2			(>100)*	58*
S3			(>100)*	61*
S4			(>100)*[c]	–
A2	70[a]	100		
S1			71/(86)*	80/60*
S2			(>100)*	55*
S3			(>100)*	62*
S4			(>100)*[c]	–
A3	65	90		
S1			70/(90)*	90
S2			(>100)*	81*
S3			(98)*	74*
S4			(>100)*[c]	–
A4	40	80		
S1			(<10)*[c]	nd
S2			(<10)*[c]	nd
S3			(<10)*[c]	nd
S4			(0)*[c]	–
A5	46	100		
S1			(39)*; 18*[b]	81*
S2			(65)*; 33*[b]	85*
S3			(67)*; 30*[b]	34*
S4			(91)*; 28*[b]	70*
A6	62	100		
S1			61	75
S2			(>100)*; 36*[b]	88*
S3			(>100)*; 49*[b]	81*
S4			(>100)*[c]	–
A7	74	100		
S1			67	68
S2			(>100)*	28*
S3			(>100)*	51*
S4			(>100)*[c]	–
A8	58	90		
S1			60	75
S2			(>100)*; 29*[b]	88*
S3			(>100)*; <5*[b]	nd
S4			(89)*; 8*[b]	nd
A9	83	77		
S1			82	90

Yields in brackets are of the crude products; yields with * correspond to results of parallel synthesis.

[a] PYBOP was used as coupling agent.

[b] Purification by SFC (supercritical fluid chromatography).

[c] Coupling product not present in the crude from MS analysis.

to slightly diminish the activity. In the series AiS1 the presence of a long alkyl spacer-arm on the piperazine motif (Y = N-$(CH_2)_{12}$-NH_2, N-$(C_2H_4O)_2$-C_2H_4-NH_2) reduced also the activity, but less than in the case of the spacer-arm fixed via an amide linkage (Y = N-CO-$(CH_2)_{11}$-NH_2). Lastly, a hydrophilic spacer of PEG type (compound A8S1) was not more appropriate than a simple alkyl chain such as aminopropyl (A9S1). Accordingly, inhibitors A9S1 and A7S1 provide good candidates for surface binding, but inhibitors A8S1 and A6S1 can also be considered. Indeed, the differences between the tested inhibitors AiSj are not so relevant to allow a deep insight on structure–activity relationship. Moreover, to favour the entry into the enzymic cavity, after inhibitor immobilization, a long spacer-arm could be more appropriate.

The micromolar activity of compounds AiSj should be sufficient to induce the expected biological response, namely the inhibition of blood coagulation, on the surface devices. Indeed, it has been previously reported that the surface grafting of simple benzamidines (which are millimolar inhibitors) could improve the hemocompatibility of polymer films [40].

After deposition of inhibitor A6S1 on the surface of a poly (ethylene terephthalate) (PET) track-etched membrane (coating at 10 nmol/cm^2), the blood clotting was reduced by 22% (Table 5); this effect corresponds to 38% of the activity of heparin coated at high concentration. The first experiments of covalent grafting of A6S1 via the hydroxyl chain-ends of the polymer furnished a material displaying about 54 pmol/cm^2 of inhibitor (calculated from the F/C atomic ratio determined by XPS). These surface modified PET membranes showed 8% inhibition of blood clotting (Table 6). Thus a coated or grafted inhibitor retains its activity and this activity is still detectable at surface concentrations in the picomolar range.

3. Conclusion

We have prepared a small library of thrombin inhibitors featuring the general structure **B** (Fig. 1). Despite the presence of a piperazinyl moiety equipped with various spacer-arms, all the compounds retained the activity of the parent structures, i.e. the corresponding piperidine, morpholine, piperazine and 4-acetyl-piperazine derivatives. This experimentally validates our design of graftable inhibitors for improving blood compatibility of polymeric materials.

First attempts to improve the blood compatibility of a polyester membrane by surface immobilization of thrombin inhibitors were encouraging as we observed a sensible decrease in clot formation. Further experiments are on the way to increase the level of surface grafting of active molecules on PET and other materials, in view to reduce more significantly the coagulation phenomenon when such devices are placed in contact with blood.

4. Experimental

4.1. Chemistry (see Tables 2 and 3)

Reagents and solvents were purchased from Acros chimica, Aldrich or Fluka. Tetrahydrofuran was dried over sodium/benzophenone, then distilled. Column chromatographies were carried out with silica gel 60 (70–230 mesh ASTM) supplied by Merck. The IR spectra were recorded with Perkin–Elmer 1710 and Shimadzu Benelux FTIR-8400S instruments, only the most significant adsorption bands being reported. The mass spectra were obtained with a Finnigan MAT TSQ-70 instrument. The high resolution mass spectra (HRMS) were performed at the University of Mons, Belgium (Prof. R. Flammang). The microanalysis was performed at the Christopher Ingold Laboratories of the University College, London (Dr. A. Stones). The melting points were determined with an

Table 3
EA/MS analysis and TLC profile of intermediates and final tested compounds (in bold)

Compound	Elemental analysis (%) or HRMS; found (calcd.)	Formula	TLC (R_f; solv.)
Boc-Arg(NO_2)-A1	425.1912 (425.1915)	$C_{16}H_{30}N_6O_5$ (+K)	0.5; CH_2Cl_2
H_2N-Arg(NO_2)-A1	309.1643 (309.1651)	$C_{11}H_{22}N_6O_3$ (+Na)	0; CH_2Cl_2
S1-Arg(NO_2)-A1	C, 42.78 (42.94); H, 4.97 (5.40); N, 16.23 (16.69); S, 6.77 (6.37)	$C_{18}H_{25}N_6O_5SF_3$ (+0.5H_2O)	0.8; MeOH/CH_2Cl_2 1:9
S2-Arg(NO_2)-A1	441.0 (54%)[a]	$C_{18}H_{29}N_6O_5S$	0.8; MeOH/CH_2Cl_2 1:9
S3-Arg(NO_2)-A1	477.1 (67%)[a]	$C_{21}H_{29}N_6O_5S$	0.8; MeOH/CH_2Cl_2 1:9
A1S1	450.1762 (450.1787)	$C_{18}H_{27}N_5O_3SF_3$	0; MeOH/CH_2Cl_2 1:9
A1S2	396.2062 (396.2069)	$C_{18}H_{30}N_5O_3S$	0; MeOH/CH_2Cl_2 1:9
A1S3	432.2059 (432.2069)	$C_{21}H_{30}N_5O_3S$	0; MeOH/CH_2Cl_2 1:9
Boc-Arg(NO_2)-A2	411.1970 (411.1968)	$C_{15}H_{28}N_6O_6$ (+Na)	0.5; CH_2Cl_2
H_2N-Arg(NO_2)-A2	289.1628 (289.1624)	$C_{10}H_{21}N_6O_4$	0; CH_2Cl_2
S1-Arg(NO_2)-A2	519.1238 (519.1250)	$C_{17}H_{23}N_6O_6SF_3$ (+Na)	0.5; MeOH/CH_2Cl_2 1:9
S2-Arg(NO_2)-A2	465.1544 (465.1532)	$C_{17}H_{26}N_6O_6S$ (+Na)	0.5; MeOH/CH_2Cl_2 1:9
S3-Arg(NO_2)-A2	501.1516 (501.1532)	$C_{20}H_{26}N_6O_6S$ (+Na)	0.6; MeOH/CH_2Cl_2 1:9
A2S1	C, 42.30 (42.38); H, 5.58 (5.80); N, 12.70 (13.00)	$C_{17}H_{24}N_5O_4SF_3$ (+AcOH·1.5H_2O)	0.6; MeOH/CH_2Cl_2 1:9
A2S2	398.1874 (398.1862)	$C_{17}H_{28}N_5O_4S$	0; MeOH/CH_2Cl_2 1:9
A2S3	434.1854 (434.1862)	$C_{20}H_{27}N_5O_4S$	0; MeOH/CH_2Cl_2 1:9
Boc-Arg(NO_2)-A3	C, 52.32 (52.18); H, 6.85 (6.63); N, 18.04 (17.98)	$C_{23}H_{35}N_7O_7$ (+0.5H_2O)	0.6; CH_2Cl_2
H_2N-Arg(NO_2)-A3	422.2172 (422.2152)	$C_{18}H_{28}N_7O_5$	0; CH_2Cl_2
S1-Arg(NO_2)-A3	C, 47.52 (47.69); H, 4.85 (4.80); N, 15.13 (15.57); S, 5.13 (5.09)	$C_{25}H_{30}N_7O_7SF_3$	0.5; IsopOH/CH_2Cl_2 1:9
S2-Arg(NO_2)-A3	576.0 (88%)[a]	$C_{25}H_{34}N_7O_7S$	0.5; IsopOH/CH_2Cl_2 1:9
S3-Arg(NO_2)-A3	612.0 (90%)[a]	$C_{28}H_{34}N_7O_7S$	0.6; IsopOH/CH_2Cl_2 1:9
A3S1	C, 38.85 (39.18); H, 6.12 (6.40); N, 13.97(14.4)	$C_{17}H_{25}N_6O_3SF_3$ (+AcOH·4H_2O)	0.6; IsopOH/CH_2Cl_2 1:9
A3S2	397.2035 (397.2022)	$C_{17}H_{29}N_6O_3S$	0; IsopOH/CH_2Cl_2 1:9
A3S3	433.2008 (433.2022)	$C_{20}H_{29}N_6O_3S$	0; IsopOH/CH_2Cl_2 1:9
Boc-Arg(NO_2)-A4	C, 48.33 (48.10); H, 8.21 (8.07); N, 23.15 (23.10)	$C_{17}H_{33}N_7O_5$(+0.5H_2O)	0.4; MeOH/CH_2Cl_2 1:9
H_2N-Arg(NO_2)-A4	316.2101 (316.2097)	$C_{12}H_{26}N_7O_3$	0; MeOH/CH_2Cl_2 1:9
S1-Arg(NO_2)-A4	524.1893 (524.1903)	$C_{19}H_{29}N_7O_5SF_3$	0.4; MeOH/CH_2Cl_2 1:9
S2-Arg(NO_2)-A4	470.2183 (470.2186)	$C_{19}H_{32}N_7O_5S$	0.4; MeOH/CH_2Cl_2 1:9
S3-Arg(NO_2)-A4	506.2170 (506.2185)	$C_{22}H_{32}N_7O_5S$	0.4; MeOH/CH_2Cl_2 1:9
Boc-Arg(NO_2)-A5	452.2234 (452.2230)	$C_{17}H_{31}N_7O_6Na$	0.5; Cyhex/EtOAc 5:4
H_2N-Arg(NO_2)-A5	330.1894 (330.1890)	$C_{12}H_{23}N_7O_4$	0; Cyhex/EtOAc 5:4
S1-Arg(NO_2)-A5	538.0 (100%)[a]	$C_{19}H_{28}N_7O_6SF_3$	0.7; MeOH/CH_2Cl_2 1:9
S2-Arg(NO_2)-A5	484.1 (100%)[a]	$C_{19}H_{30}N_7O_6S$	0.7; MeOH/CH_2Cl_2 1:9
S3-Arg(NO_2)-A5	520.1 (90%)[a]	$C_{22}H_{30}N_7O_6S$	0.7; MeOH/CH_2Cl_2 1:9
S4-Arg(NO_2)-A5	521.1 (100%)[a]	$C_{21}H_{29}N_8O_6S$	0.7; MeOH/CH_2Cl_2 1:9
A5S1	493.1841 (493.1845)	$C_{19}H_{28}N_6O_4SF_3$	0; MeOH/CH_2Cl_2 1:9
A5S2	439.2131 (439.2128)	$C_{19}H_{31}N_6O_4S$	0; MeOH/CH_2Cl_2 1:9
A5S3	475.2121 (475.2128)	$C_{22}H_{31}N_6O_4S$	0; MeOH/CH_2Cl_2 1:9
A5S4	480.2386 (480.2393)	$C_{21}H_{34}N_7O_4S$	0; MeOH/CH_2Cl_2 1:9
Boc-Arg(NO_2)-A6	C, 55.70 (55.39); H, 8.00 (7.97); N, 16.14 (16.61)	$C_{35}H_{59}N_9O_9$ (+0.5H_2O)	0.5; EtOAc/isopOH 1:1
H_2N-Arg(NO_2)-A6	650.4001 (650.3990)	$C_{30}H_{52}N_9O_7$	0; EtOAc/isopOH 1:1
S1-Arg(NO_2)-A6	880.3596 (880.3615)	$C_{37}H_{54}N_9O_9SF_3$ (+Na)	0.4; IsopOH/CH_2Cl_2 1:9
S2-Arg(NO_2)-A6	804.2 (5%)	$C_{37}H_{58}N_9O_9SF_3$	0.4; IsopOH/CH_2Cl_2 1:9
S3-Arg(NO_2)-A6	840.2 (5%)	$C_{40}H_{58}N_9O_9S$	0.5; IsopOH/CH_2Cl_2 1:9
A6S1	634.3737 (634.3726)	$C_{29}H_{51}N_7O_3SF_3$	0; IsopOH/CH_2Cl_2 1:9
A6S2	580.4003 (580.4009)	$C_{29}H_{54}N_7O_3S$	0; IsopOH/CH_2Cl_2 1:9
A6S3	616.3994 (616.4009)	$C_{32}H_{54}N_7O_3S$	0; IsopOH/CH_2Cl_2 1:9
Boc-Arg(NO_2)-A7	786.4150 (786.4126)	$C_{35}H_{57}N_9O_{10}$ (+Na)	0.8; IsopOH/CH_2Cl_2 1:9
H_2N-Arg(NO_2)-A7	664.3768 (664.3782)	$C_{30}H_{50}N_9O_8$	0; IsopOH/CH_2Cl_2 1:9
S1-Arg(NO_2)-A7	C, 50.17 (50.97); H, 5.94 (6.01); N, 14.0 (14.46)	$C_{37}H_{52}N_9O_{10}SF_3$	0.8; IsopOH/CH_2Cl_2 1:9
S2-Arg(NO_2)-A7	818.1 (5%)[a]	$C_{37}H_{56}N_9O_{10}S$	0.5; IsopOH/CH_2Cl_2 1:9
S3-Arg(NO_2)-A7	854.1 (33%)[a]	$C_{40}H_{56}N_9O_{10}S$	0.5; IsopOH/CH_2Cl_2 1:9
A7S1	C, 50.00 (50.43); H, 7.36 (7.44); N, 12.32 (12.48)	$C_{29}H_{48}N_7O_4SF_3$ (+2AcOH·H_2O)	0; IsopOH/CH_2Cl_2 1:9
A7S2	594.3793 (594.3802)	$C_{29}H_{52}N_7O_4S$	0; IsopOH/CH_2Cl_2 1:9
A7S3	630.3795 (630.3802)	$C_{32}H_{51}N_7O_4S$	0; IsopOH/CH_2Cl_2 1:9
Boc-Arg(NO_2)-A8	C, 49.75 (49.92); H, 6.94 (6.79); N, 17.43 (18.01)	$C_{29}H_{47}N_9O_{11}$	0.3; IsopOH/CH_3CN 2:8
H_2N-Arg(NO_2)-A8	598.2949 (598.2949)	$C_{24}H_{40}N_9O_9$	0; IsopOH/CH_3CN 2:8
S1-Arg(NO_2)-A8	806.2734 (806.2755)	$C_{31}H_{43}N_9O_{11}SF_3$	0.5; IsopOH/CH_2Cl_2 1:9
S2-Arg(NO_2)-A8	752.2 (5%)	$C_{31}H_{46}N_9O_{11}S$	0.5; IsopOH/CH_2Cl_2 1:9
S3-Arg(NO_2)-A8	788.1 (10%)	$C_{34}H_{46}N_9O_{11}S$	0.5; IsopOH/CH_2Cl_2 1:9
S4-Arg(NO_2)-A8	789.1 (5%)	$C_{33}H_{45}N_{10}O_{11}S$	0.5; IsopOH/CH_2Cl_2 1:9
A8S1	582.2709 (582.2685)	$C_{23}H_{39}N_7O_5SF_3$	0; IsopOH/CH_2Cl_2 1:9

(*continued on next page*)

Table 3 (*continued*)

Compound	Elemental analysis (%) or HRMS; found (calcd.)	Formula	TLC (R_f; solv.)
A8S2	528.2935 (528.2968)	$C_{23}H_{42}N_7O_5S$	0; IsopOH/CH_2Cl_2 1:9
Boc-Arg(NO_2)-A9	C, 48.62 (48.66); H, 6.16 (6.76); N, 19.05 (19.65)	$C_{26}H_{41}N_9O_9$ (+H_2O)	0.1; IsopOH/CH_2Cl_2 1:9
H_2N-Arg(NO_2)-A9	C, 48.14 (48.18); H, 6.43 (6.35); N, 24.15 (24.08)	$C_{21}H_{33}N_9O_7$	0; IsopOH/CH_2Cl_2 1:9
S1-Arg(NO_2)-A9	C, 46.06 (45.96); H, 5.09 (4.96); N, 16.98 (17.23)	$C_{28}H_{36}N_9O_9SF_3$	0.5; IsopOH/CH_2Cl_2 1:9
A9S1	C, 44.37 (44.03); H, 6.32 (6.62); N, 14.68 (14.98)	$C_{20}H_{32}N_7O_3SF_3$ (+2AcOH·1.5H_2O)	0; IsopOH/CH_2Cl_2 1:9

[a] MS (APCI) *m/z* (rel. ab %).

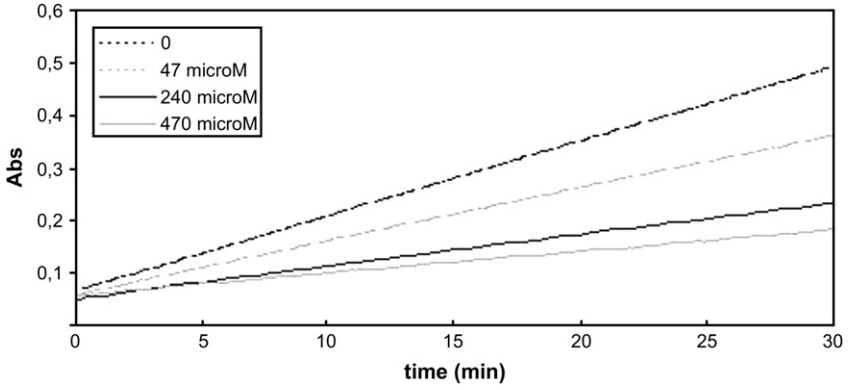
Electrothermal microscope and are uncorrected. The ^{1}H and ^{13}C NMR spectra were recorded on Varian Gemini 300 (at 300 MHz for proton and 75 MHz for carbon) or Bruker AM-500 spectrometers (at 500 MHz for proton and 125 MHz for carbon); the chemical shifts were reported in ppm (δ) downfield from tetramethylsilane (TMS).

4.1.1. Preparation of the spacer-arms (A7 and A8)

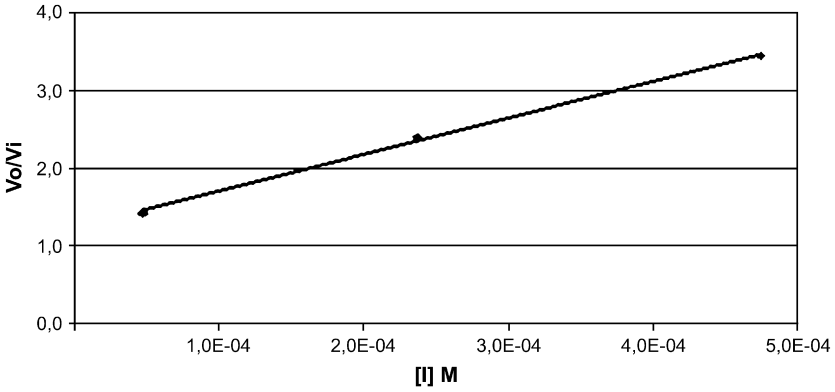
The preparation of the spacer-arms A6 and A9 has been described elsewhere [41].

4.1.1.1. 12-(4-Nitro-benzyloxycarbonylamino)-dodecanoic acid (Scheme 2, part 1). 12-Amino dodecanoic acid (250 mg, 1.16 mmol, 1 equiv) was dissolved in THF (3 mL), water (3 mL) and NaOH 1 N (1.14 mL, 1 equiv). 4-Nitrobenzyl chloroformate (246 mg, 1.16 mmol, 1 equiv) in THF (1 mL) and NaOH 1 N (1.3 mL, 1.1 equiv) were added simultaneously to the reaction mixture at 0 °C. The solution was stirred at 0 °C for 10 min and then for 2 h at room temperature, checking pH to be above 10. HCl 1 N was added to the reaction mixture to reach pH 1, and the solution was then extracted twice with EtOAc. The organic layers were combined, washed with HCl 1 N and water, dried over $MgSO_4$, and evaporated under reduced pressure to give a yellow solid that was purified by column chromatography on SiO_2 to obtain pure carbamate (0.345 g) as a white solid (75% yield). Mp = 107.9–108.9 °C; R_f = 0.3 (SiO_2, EtOAc/cyclohexane 3:5, UV); IR (CH_2Cl_2 liquid film on NaCl) ν_{max} (cm^{-1}): 3367, 2920, 2851, 1725, 1689, 1613, 1527, 1351, 1256, 1246; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.27 (14H, m), 1.52 (2H, m), 1.64 (2H, m), 2.36 (2H, t, J = 7.1 Hz), 3.21 (2H, m), 4.84 (1H, m), 5.20 (2H, s), 7.52 (2H, d, J = 8.6 Hz), 8.22 (2H, d, J = 8.6 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 24.7, 26.7, 29.0, 29.1, 29.2, 29.3, 29.4, 29.9, 33.8, 41.3, 65.1, 123.7, 128.0, 144.1, 150.2, 155.7, 178.2; MS (APCI) *m/z* (ass, rel. ab. %): 395.0 (M + 1, 100); Anal. calcd. for $C_{20}H_{30}N_2O_6$: C, 60.90; H, 7.65; N, 7.10%. Found: C, 60.55; H, 7.45; N, 6.76%.

4.1.1.2. 4-[12-(4-Nitro-benzyloxycarbonylamino)-dodecanoyl]-piperazine-1-carboxylic acid tert-butyl ester (Scheme 2, part 1). 12-(4-Nitro-benzyloxycarbonylamino)-dodecanoic acid (3.0 g, 7.8 mmol, 1 equiv) was dissolved in dry THF (120 mL) and NEt_3 (1.095 g, 7.8 mmol, 1 equiv). The solution was cooled down to −25/−20 °C and then isobutyl chloroformate (1 mL, 7.8 mmol, 1 equiv) was added dropwise. The solution was stirred for 15 min at −20/−25 °C and then *tert*-butyl-piperazine-1-carboxylate (1.432 g, 7.8 mmol, 1 equiv) was added dropwise to the reaction mixture that was stirred for additional 20 min at low temperature and then 2 h at room temperature. The solution was concentrated under reduced pressure and the solid residue was extracted and partitioned between EtOAc and brine. The organic layer was then washed with water, dried over $MgSO_4$ and evaporated under reduced pressure. The pure product was isolated by column chromatography purification (3.0 g, 70% yield). Mp = 71.9–72.3 °C; R_f = 0.5 (SiO_2, CH_2Cl_2/isopropanol 99:1, UV); IR (CH_2Cl_2 liquid film on NaCl) ν_{max} (cm^{-1}): 3300, 2916, 2848, 1713, 1698, 1633, 1528, 1418, 1351, 1265, 1232, 1160, 1132; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.26 (m, 14H), 1.47 (s, 9H), 1.61 (m, 4H), 2.32 (m, 2H), 3.19 (q, 2H, J = 6.8 Hz), 3.43 (m, 6H), 3.58 (m, 2H), 4.83 (m, 1H), 5.18 (s, 2H), 7.51 (d, 2H,



Graph 1. *p*-Nitroaniline release versus time monitored at 405 nm. Effect of inhibitor (compound **A1S1**) concentration on substrate S-2238 hydrolysis.



Graph 2. Velocity ratio versus inhibitor concentration. Linear fitting for K_i calculation ($R^2 = 0.9984$).

$J = 8.7$ Hz), 8.21 (d, 2H, $J = 8.7$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 25.3, 26.7, 28.4, 29.2, 29.4, 29.9, 33.3, 41.3, 43.6, 45.4, 65.0, 80.2, 123.7, 128.1, 144.2, 147.3, 154.6, 155.8, 171.8; MS (APCI) *m/z* (ass, rel. ab. %): 563.0 (M + 1, 75), 507.1 (M − 55, 100); Anal. calcd. for $C_{29}H_{46}N_5O_7$: C, 61.90; H, 8.24; N, 9.96%. Found: C, 61.68; H, 8.35; N, 9.81%.

4.1.1.3. (12-Oxo-12-piperazin-1-yl-dodecyl)-carbamic acid 4-nitro-benzyl ester (A7). 4-[12-(4-Nitro-benzyloxycarbonylamino)-dodecanoyl]-piperazine-1-carboxylic acid *tert*-butyl ester (3 g, 5.33 mmol, 1 equiv) was dissolved in CH_2Cl_2 (100 mL) and TFA (0.912 g, 8.0 mmol, 1.5 equiv). The reaction mixture was stirred at room temperature for 4 h. The solvent was then removed by rotary evaporation at reduced pressure and the solid residue was triturated and suspended in Et_2O to obtain the desired product as trifluoroacetate salt (3.06 g, quantitative yield). $R_f = 0$ (SiO_2, CH_2Cl_2/isopropanol 99:1, UV); IR (CH_2Cl_2 liquid film on NaCl), ν_{max} (cm^{-1}): 3328, 2926, 2854, 1677, 1523, 1347, 1252, 1202, 1178; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.25 (m, 14H), 1.49 (m, 2H), 1.60 (m, 2H), 2.32 (m, 2H), 3.17 (m, 6H), 3.77 (m, 2H), 3.88 (m, 2H), 4.86 (br, 1H), 5.17 (s, 2H), 7.51 (d, 2H, $J = 8.7$ Hz), 8.20 (d, 2H, $J = 8.7$ Hz), 10.0 (br, 2H); ^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm): 25.0, 26.7, 29.2, 29.3, 29.4, 29.9, 32.9, 43.4, 65.1, 123.7, 128.1, 144.1, 147.2, 155.8, 171.7; MS (APCI), *m/z* (ass, rel. ab. %): 925 (M·M + 1, 12), 463.3 (M + 1, 100); Anal. calcd. for $C_{26}H_{39}N_4F_3O_7$: C, 54.16; H, 6.82; N, 9.72%. Found: C, 53.77; H, 6.84; N, 9.54%.

4.1.1.4. 2-[2-(2-Azido-ethoxy)-ethoxy]-ethanol (Scheme 2, part 2). 2-[2-(2-Chloroethoxy)-ethoxy]-ethanol (5.0 g, 29.7 mmol, 1 equiv) was dissolved in water (30 mL). NaI (0.9 g, 6 mmol, 0.2 equiv) and NaN_3 (20 g, 0.3 mol, 10.4 equiv) were added in one portion and the solution was stirred at 50 °C for 54 h. The mixture was filtered and extracted four times with EtOAc. The aqueous phase was saturated with NaCl and extracted two times with EtOAc. The organic layers were combined, evaporated under reduced pressure to give the pure azide (5.016 g) as a yellow liquid (97% yield). $R_f = 0.5$ (SiO_2, AcOEt, UV); IR (CH_2Cl_2 liquid film on NaCl) ν_{max} (cm^{-1}) 3415, 2873, 2359, 2338, 2109, 1653, 1458, 1300, 1119, 1070, 933; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 2.60 (br, 1H), 3.36 (t, 2H, $J = 5.2$ Hz), 3.59 (m, 2H), 3.65 (m, 6H), 3.72 (m, 2H); ^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm): 51.0, 62.3, 70.6, 70.9, 71.9, 73.1; MS (APCI), *m/z* (ass, rel. ab. %): 148.1 ((M − N_2) + 1, 100).

Table 4
Inhibition constants K_i in μM (compound code in brackets)

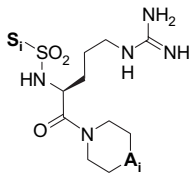
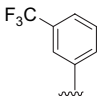
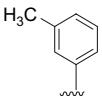
				
CH_2	2.3–2.6 (**A1S1**)	0.9–3.2 (**A1S2**)	0.7–1.8 (**A1S3**)	–
O	0.9–1.8 (**A2S1**)	1.5–2.6 (**A2S2**)	0.4–0.9 (**A2S3**)	–
NH	5.6–5.7 (**A3S1**)	5.4–6.0 (**A3S2**)	6.4–6.7 (**A3S3**)	–
N-CO-CH_3	2.5–2.8 (**A5S1**)	4.4–5.3 (**A5S2**)	1.0–1.2 (**A5S3**)	1.2–2.0 (**A5S4**)
N-$(CH_2)_{12}$-NH_2	7.3–8.2 (**A6S1**)	1.0–2.2 (**A6S2**)	5.3–5.8 (**A6S3**)	–
N-CO-$(CH_2)_{11}$-NH_2	2.5–5.7 (**A7S1**)	3.7–4.5 (**A7S2**)	8.0–8.3 (**A7S3**)	–
N-$(C_2H_4$-O$)_2$-C_2H_4-NH_2	11.5–15.3 (**A8S1**)	–	–	–
N-$(CH_2)_3$-NH_2	1.9–5.7 (**A9S1**)	–	–	–

Table 5
Inhibitor coating effect on blood clot formation

Surface[a]	Inhibitor concentration	Clot weight[b] (mg)	% Inhibition	Relative activity (%)
PET, native membrane	–	27.5 ± 1.5	–	
PET, coated with heparin	500 U[c]/cm^2	11.4 ± 0.5	58	100
PET, coated with **A6S1**	10 nmol/cm^2	21.6 ± 3.0	22	38

[a] PET samples are disks of 2.6 cm of diameter, placed in contact with human blood as described in Section 4.
[b] Results are the mean of three independent experiments ± standard deviation.
[c] USP heparin units.

4.1.1.5. 2-[2-(2-Amino-ethoxy)-ethoxy]-ethanol (Scheme 2, part 2). 2-[2-(2-Azido-ethoxy)-ethoxy]-ethanol (5.246 g, 29.9 mmol, 1 equiv) was dissolved in MeOH (60 mL). Pd/C (0.9 g, 0.15 mmol, 0.005 equiv) was added, then the flask was purged with argon and filled with hydrogen. The solution was stirred 24 h at room temperature. The mixture was filtered and the solvent evaporated at reduced pressure to obtain the pure amino alcohol (0.4266 g, 95% yield). $R_f = 0$ (SiO_2, AcOEt, UV); IR (KBr) ν_{max} (cm^{-1}): 3383, 3053, 2873, 2305, 2253, 1603, 1458, 1422, 1350, 1265, 1116, 1072, 909, 735; ^{1}H NMR (CD_3OD/300 MHz) δ (ppm): 2.92 (t, 2H, $J = 5.2$ Hz), 3.59 (t, 2H, $J = 5.2$ Hz), 3.61 (m, 6H), 3.66 (m, 2H) 3.73 (t, 2H, $J = 4.5$ Hz); ^{13}C NMR (50 MHz, $CDCl_3$) δ (ppm): 40.9, 61.0, 70.0, 70.1, 71.6, 72.6; MS (APCI), *m/z* (ass, rel. ab. %): 150.2 (M + 1, 100).

4.1.1.6. {2-[2-(2-Hydroxy-ethoxy)-ethoxy]-ethyl}-carbamic acid 4-nitro-benzyl ester (Scheme 2, part 2). 2-[2-(2-Amino-ethoxy)-ethoxy]-ethanol (3.601, 24.1 mmol, 1 equiv) was dissolved in distilled CH_2Cl_2 (100 mL) and the mixture was cooled down to 0 °C. A solution of *p*-nitrobenzyl chloroformate (5.363 g, 24.1 mmol, 1 equiv) dissolved in CH_2Cl_2 (20 mL) and NEt_3 (2.699 g, 24.1 mmol, 1 equiv) was added dropwise and simultaneously to the reaction mixture. The solution was stirred 15 min at 0 °C and additional 2 h at room temperature. The mixture was then washed twice with HCl 1 N, once with water and NaOH 1 N, and finally with brine. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure. The product was crystallized from a solution of pentane/CH_2Cl_2 1:1, filtered and washed with cold pentane to give the pure carbamic ester (5.447 g, 69% yield). Mp: 81–84 °C; $R_f = 0.1$ (SiO_2, EtOAc, UV); IR (KBr) ν_{max} (cm^{-1}): 3428, 3334, 2933, 2873, 1721, 1607, 1521, 1453, 1347, 1256, 1110, 1059, 910, 735; ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 3.41 (q, 2H, $J = 5.3$ Hz), 3.58 (q, 2H, $J = 5.3$ Hz), 3.60 (m, 2H), 3.65 (m, 4H), 3.73 (m, 2H), 5.19 (s, 2H), 5.53 (t, 1H), 7.51 (d, 2H, $J = 8.8$ Hz), 8.21 (d, 2H, $J = 8.8$ Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 40.84, 61.61, 65.01, 69.85, 70.25, 72.38, 123.6, 128.00, 143.97, 147.45, 155.81; MS (APCI), *m/z* (ass, rel. ab. %): 329.0 (M + 1, 37); Anal. calcd. for $C_{14}H_{20}N_2O_7$: C, 51.22; H, 6.14; N, 8.53%. Found: C, 51.36; H, 6.24; N, 8.39%.

Table 6
Inhibitor grafting effect on blood clot formation

Surface[a]	Inhibitor concentration (by XPS)	Clot weight[c] (mg)	% Inhibition
PET, blank[b]	<0.5% (adsorption)	29.7 ± 0.4	–
PET, grafted with **A6S1**	1.9% or 54 pmol/cm^2	27.5 ± 0.5	8

[a] See Table 5.
[b] This sample has been exposed to all the chemical treatments, but with the omission of the surface activating agent, i.e. tosyl chloride.
[c] See Table 5.

4.1.1.7. Methanesulfonic acid 2-{2-[2-(4-nitro-benzyloxycarbonylamino)-ethoxy]-ethoxy}-ethyl ester (Scheme 2, part 2). The *N*-protected alcohol (5.375 g, 16.38 mmol, 1 equiv) was dissolved in distilled CH_2Cl_2 (50 mL) and the solution was cooled down to 0 °C. Pyridine (2.589 g, 32.76 mmol, 2 equiv) and methanesulfonyl chloride (2.800 g, 24.57 mmol, 1.5 equiv), both diluted in CH_2Cl_2 (5 mL) were added dropwise to the reaction mixture. After stirring at room temperature for 24 h, the solution was washed with HCl 1 N, then a saturated solution of $NaHCO_3$ and brine. The organic phase was dried over $MgSO_4$, concentrated under reduced pressure and the liquid residue was purified by flash chromatography to obtain the mesylate (5.990 g) as yellow gel (90% yield). $R_f = 0.3$ (SiO_2, CH_2Cl_2/EtOAc 7:3, UV); IR (KBr) ν_{max} (cm^{-1}): 3393, 2946, 2872, 1733, 1717, 1700, 1607, 1521, 1540, 1507, 1347, 1248, 1172, 1108, 1014, 973, 920, 854, 801, 775, 738; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 2.02 (s, 3H), 3.39 (dt, 2H, $J = 4.8$, 5.7 Hz), 3.55 (q, 2H, $J = 4.8$ Hz), 3.62 (m, 2H), 3.64 (m, 2H), 3.75 (tt, 2H, $J = 3.8$, 4.8 Hz), 4.36 (tt, 2H, $J = 3.8$, 4.8 Hz), 5.18 (s, 2H), 5.43 (t, 1H, $J = 5.7$ Hz), 7.50 (d, 2H, $J = 8.7$ Hz), 8.19 (d, 2H, $J = 8.7$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 38.1, 41.4, 65.5, 69.4, 69.5, 70.4, 70.6, 71.0, 124.2, 128.5, 144.5, 147.8, 156.3; MS (APCI), *m/z* (ass, rel. ab. %): 407.0 (M + 1, 100); Anal. calcd. for $C_{15}H_{22}N_2O_9S$: C, 44.33; H, 5.46; N, 6.89%. Found: C, 44.09; H, 5.54; N, 6.47%.

4.1.1.8. 4-(2-{2-[2-(4-Nitro-benzyloxycarbonylamino)-ethoxy]-ethoxy}-ethyl)-piperazine-1-carboxylic acid tert-butyl ester (Scheme 2, part 2). The mesylate (5.90 g, 14.52 mmol, 1 equiv) was dissolved in CH_3CN (50 mL). NaI (2.18 mmol, 14.52 mmol, 1 equiv) was dissolved in H_2O (1.5 mL) and added to the mesylate solution that was stirred for 30 min at 85 °C. Boc-piperazine (2.71 g, 14.52 mmol, 1 equiv) dissolved in DIPEA (1.87 g, 14.52 mmol, 1 equiv) and CH_3CN (5 mL) was added in one portion and the solution was stirred for 24 h at room temperature. Then the solution was concentrated, the solid residue dissolved in AcOEt and washed with brine. The organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash chromatography to give the desired product as yellow gel (7.21 g, 92% yield).

$R_f = 0.5$ (SiO_2, CH_2Cl_2/isopropanol 9:1, UV); IR (CH_2Cl_2 liquid film on NaCl) ν_{max} (cm^{-1}): 3310, 2936, 2868, 1725, 1691, 1606, 1523, 1458, 1422, 1365, 1347, 1247, 1170, 1130, 1005, 937, 859, 803, 771, 738; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.44 (s, 9H), 2.49 (m, 4H), 2.64 (m, 2H), 3.40 (dt, 2H, $J = 5.0$, 5.3 Hz), 3.45 (m, 4H), 3.56 (q, 2H, $J = 5.3$ Hz), 3.60 (m, 4H), 3.65 (m, 2H), 5.19 (s, 2H), 5.54 (t, 1H, $J = 5.0$ Hz), 7.51 (d, 2H, $J = 8.8$ Hz), 8.21 (d, 2H, $J = 8.8$ Hz), ^{13}C NMR (75 MHz, $CDCl_3$) δ (ppm): 28.3, 40.9, 42.6, 43.6, 53.2, 57.6, 65.0, 68.4, 69.8, 70.1, 70.2, 79.7, 123.6, 128.0, 144.0, 147.5, 154.5, 155.9; MS (APCI), *m/z* (ass, rel. ab. %): 497.2 (M + 1, 100); Anal. calcd. for $C_{23}H_{36}N_4O_8 \cdot 0.5H_2O$: C, 54.64; H, 7.38; N, 11.08%. Found: C, 54.53; H, 7.40; N, 10.93%.

4.1.1.9. {2-[2-(2-Piperazin-1-yl-ethoxy)-ethoxy]-ethyl}-carbamic acid 4-nitro-benzyl ester (A8). The *N*-protected compound (4.39 g, 8.84 mmol, 1 equiv) was dissolved in a 1:9 mixture of TFA/CH_2Cl_2 (7 mL) and the solution was stirred at room temperature for 4 h. The solution was concentrated under reduced pressure to give a brown gel that was triturated and suspended in Et_2O until precipitation of a brown solid occurred. The solid residue was dissolved in water and basified to pH 12 with a 1 N NaOH solution that was saturated with NaCl, and extracted four times with CH_2Cl_2. The organic layers were combined, dried over $MgSO_4$ and concentrated at reduced pressure to give the pure deprotected product (3.339 g) as a brown gel (95% recovery yield). $R_f = 0$ (SiO_2, CH_2Cl_2/isopropanol 9:1, UV); IR (CH_2Cl_2 liquid film on NaCl) ν_{max} (cm^{-1}): 3326, 2940, 2870, 1718, 1606, 1521, 1458, 1347, 1260, 1134, 1105, 854, 739; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 2.47 (m, 4H), 2.59 (t, 2H, $J = 5.7$ Hz), 2.89 (m, 4H, $J = 4.8$ Hz), 3.40 (q, 2H, $J = 5.3$ Hz), 3.57 (t, 2H, $J = 5.7$ Hz), 3.61 (m, 6H, $J = 5.3$ Hz), 5.20 (s, 2H), 5.62 (t, 1H, $J = 5.0$ Hz), 7.52 (d, 2H, $J = 8.8$ Hz), 8.22 (d, 2H, $J = 8.8$ Hz); HRMS-ESI, *m/z*: 397.2072 (calcd. for $C_{18}H_{29}N_4O_6 = 397.2087$).

4.1.2. General procedure for the preparation of 5-(3-nitro-guanidino)-2(S)-tert-butoxycarbonylamino-1-(azacyclo-hexan-1-yl)pentan-1-one (Boc-Arg(NO_2)-Ai; Scheme 1, step i).

N-α-Boc-*N*-α-nitro-L-arginine (1.00 g, 3.13 mmol, 1 equiv) was dissolved in dry THF (10 mL) and NMM (0.64 g, 6.26 mmol, 2 equiv). Isobutyl chloroformate (0.43 g, 3.13 mmol, 1 equiv) was then added dropwise at −20/−25 °C. After 30 min azacyclohexane (3.13 mmol, 1 equiv) in dry THF (2 mL) was added dropwise at −20 °C and stirred for additional 20 min. The solution was allowed to warm up to room temperature and stirred for 1 h. The solvent was then evaporated and the solid residue partitioned between EtOAc and brine. The organic layer was dried over $MgSO_4$ and concentration under vacuum gave crude product that was either submitted directly to deprotection, or purified by column chromatography on SiO_2.

Compounds Boc-Arg(NO_2)-Ai with i being 1, 3, 6 and 9 have been described elsewhere [41]. R_f, MS/EA for all following compounds are reported in Table 3.

4.1.2.1. [(S)-4-(N′-Nitro-guanidino)-1-(morpholine-4-carbonyl)-butyl]-carbamic acid tert-butyl ester (Boc-Arg(NO_2)-A2). *N*-α-Boc-*N*-α-nitro-L-arginine (0.500 g, 1.56 mmol, 1 equiv) and PyBop (0.817 g, 1.56 mmol, 1 equiv) were dissolved in dry CH_2Cl_2 (8 mL). Morpholine (0.545 g, 6.26 mmol, 4 equiv) was dissolved in dry CH_2Cl_2 (2 mL) and added to the reaction mixture with stirring at room temperature. After 1 h 30 min the solution was filtered to eliminate the white precipitate, and then washed once with NaOH 1 N. The organic layer was concentrated at reduced pressure to give a brown gel (0.9 g) containing 56% of desired product (84% raw yield, by NMR). The solid residue can be submitted directly to the next reaction or purified by column chromatography (0.452 g, 70% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.42 (s, 9H), 1.64 (m, 2H), 1.73 (m, 2H), 3.22 (m, 1H), 3.47 (m, 2H), 3.59 (m, 2H), 3.67 (m, 5H), 4.55 (m, 1H), 5.80 (m, 1H), 7.80 (s, 2H), 8.95 (s, 1H); IR (CH_2Cl_2 liquid film on NaCl) ν_{max} (cm^{-1}): 3310, 2961, 2922, 2847, 1701, 1626, 2529, 1446, 1371, 1270, 1164, 1114, 847.

4.1.2.2. [1-(4-Ethyl-piperazine-1-carbonyl)-4-(N′-methyl-guanidino)-butyl]-carbamic acid tert-butyl ester (Boc-Arg(NO_2)-A4). This product was obtained from 5.0 g (15.7 mmol, 1 equiv) of *N*-Boc-*N*-nitro-arginine, 3.18 g (31.3 mmol, 2 equiv) of NMM, 2.14 g (15.7 mmol, 1 equiv) of isobutyl chloroformate, 1.78 g (15.7 mmol, 1 equiv) of ethyl piperazine and again 1.60 g (15.7 mmol, 1 equiv) of NMM in 80 mL of THF. The solid residue obtained after workup was purified by two column chromatographies (CH_3CN/isopropanol 9:1, CH_3CN/isopropanol 5:5) to obtain 2.61 g of an orange gel (yield 40%). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.11 (t, 3H, $J = 7.1$ Hz), 1.42 (s, 9H), 1.64 (m, 7H), 1.74 (m, 8H), 2.48 (m, 6H), 3.27 (m, 1H), 3.48 (m, 2H), 3.58 (m, 1H), 3.66 (m, 2H), 4.56 (m, 1H), 5.84 (d, 1H, 7.5 Hz), 7.81 (br, 2H), 8.89 (br, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 11.58, 24.27, 28.28, 31.20, 40.18, 41.88, 45.22, 48.54, 52.00, 52.46, 80.37, 156.54, 159.30, 169.74; IR (CH_2Cl_2 liquid film on NaCl) ν_{max} (cm^{-1}): 3400, 2976, 2938, 2826, 1992 (C═O st), 1634 (C═N), 1531 (N═O as), 1455, 1255, 1165.

4.1.2.3. [(S)-1-(4-Acetyl-piperazine-1-carbonyl)-4-(N′-nitro-guanidino)-butyl]-carbamic acid tert-butyl ester (Boc-Arg(NO_2)-A5). This product was obtained from 5.0 g (15.7 mmol, 1 equiv) of *N*-Boc-*N*-nitro-arginine, 3.18 g of NMM (31.3 mmol, 2 equiv), 2.14 g (15.7 mmol, 1 equiv) of isobutyl chloroformate, 2.0 g (15.7 mmol, 1 equiv) of acetyl piperazine and again 1.60 g (15.7 mmol, 1 equiv) of NMM in 80 mL of THF. The solid residue was purified by column chromatography to obtain 3.26 g of a white solid (46% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.41 (s, 9H), 1.64 (m, 2H), 1.73 (m, 2H), 2.10 (s, 3H), 3.48 (m, 2H), 3.10−3.80 (m, 8H), 4.59 (m, 1H), 5.80 (m, 1H), 7.73 (br, 2H), 8.82 (br, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 21.15, 21.21, 24.27, 28.20, 30.74, 40.31, 40.82, 41.14, 45.61, 45.92, 41.77, 41.94, 45.00, 45.26, 48.88, 80.27, 156.20, 159.30, 169.20, 169.24, 170.26, 170.41; IR (CH_2Cl_2 liquid film on

NaCl) ν_{max} (cm^{-1}): 3420, 2976, 2932, 2870, 1705, 1622, 1521, 1436, 1366, 1253, 1166.

4.1.2.4. (12-{4-[(S)-2-tert-Butoxycarbonylamino-5-(N′-nitro-guanidino)-pentanoyl]-piperazin-1-yl}-12-oxo-dodecyl)-carbamic acid 4-nitro-benzyl ester (Boc-Arg(NO2)-A7). This product was prepared from *N*-Boc-*N*-nitro-arginine (1.71 g, 5.34 mmol, 1 equiv), NMM (1.62 g, 16 mmol, 3 equiv), isobutyl chloroformate (0.73 g, 5.34 mmol, 1 equiv) and (12-oxo-12-piperazin-1-yl-dodecyl)-carbamic acid 4-nitro-benzyl ester (3.08 g, 5.34 mmol, 1 equiv) to give a brown solid (3.67 g) containing the desired product (83% by NMR, 75% raw yield). The raw product could be submitted to the next reaction step without further purifications. Pure product was obtained by column chromatography (74% isolated yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.26 (br, 12H), 1.43 (s, 9H), 1.49 (m, 2H), 1.64 (m, 4H), 1.75 (m, 2H), 2.33 (t, 2H, $J = 7.2$ Hz), 3.18 (q, 2H, $J = 6.6$ Hz), 3.26 (m, 1H), 3.48 (m, 6H), 3.70 (m, 2H), 4.60 (m, 1H), 4.85 (m, 1H), 5.18 (s, 2H), 5.85 (dd, 1H, $J = 8.2$ Hz), 7.50 (d, 2H, $J = 8.7$ Hz), 7.65 (br, 2H), 8.20 (d, 2H, $J = 8.7$ Hz), 8.80 (br, 1H).

4.1.2.5. (12-{4-[(S)-2-tert-Butoxycarbonylamino-5-(N′-nitro-guanidino)-pentanoyl]-piperazin-1-yl}-12-oxo-dodecyl)-carbamic acid 4-nitro-benzyl ester (Boc-Arg(NO2)-A8). This product was obtained from 1.84 g (5.78 mmol, 1 equiv) of *N*-Boc-*N*-nitro-arginine, 1.17 g (11.6 mmol, 2 equiv) of NMM, 0.79 g (5.8 mmol, 1 equiv) of isobutyl chloroformate, 2.29 g (5.8 mmol, 1 equiv) of product **A8** and again 0.58 g (5.8 mmol, 1 equiv) of NMM in 20 mL of THF. The solid residue was purified by two column chromatographies (EtOAc/isopropanol 8:2, and then CH_3CN/isopropanol 6:4) in order to obtain 2.32 g of pure product (58% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.43 (s, 9H), 1.62 (m, 2H), 1.73 (m, 2H), 2.53 (m, 4H), 2.64 (m, 2H), 3.26 (m, 1H), 3.39 (m, 2H), 3.44 (m, 2H), 3.56 (m, 2H), 3.61 (m, 9H), 4.55 (m, 1H), 5.19 (s, 2H), 5.48 (m, 1H), 5.82 (m, 1H), 7.51 (d, 2H, $J_{HH} = 8.6$ Hz), 7.79 (br, 2H), 8.20 (d, 2H, $J_{HH} = 8.6$ Hz), 8.86 (br, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 24.2, 28.3, 31.3, 40.1, 40.9, 41.8, 45.1, 48.5, 52.8, 53.2, 57.3, 65.0, 68.5, 69.8, 70.1, 70.2, 80.4, 123.6, 128.0, 144.0, 147.4, 155.9, 156.6, 159.3, 169.7; IR (CH_2Cl_2 liquid film on NaCl) ν_{max} (cm^{-1}) 3328, 2930, 2870, 1707, 1628, 1518, 1451, 1341, 1252, 1158, 1100, 723.

4.1.3. General procedure for the preparation of 2(S)-amino-5-nitro-guanidino-1-(azacyclohexan-1-yl)-pentan-1-one (H2N-Arg(NO2)-Ai; Scheme 1, step ii).

Product Boc-Arg(NO_2)-Ai (1.25 mmol) was dissolved in a 1:1 mixture of TFA/CH_2Cl_2 (10 mL) and stirred at room temperature for 3 h. The solution was concentrated at reduced pressure and the obtained orange gel was suspended and triturated in Et_2O until precipitation of the trifluoroacetate salt as a crystalline solid occurred. The precipitate was washed with Et_2O and used directly in the next reaction (quantitative yield). For spectroscopic analysis the product was converted to the free amine by dissolution in NaOH 1 N, and extraction of the aqueous solution with EtOAc (about 90% product recovery).

Compounds H_2N-Arg(NO_2)-Ai with i being 1, 3, 6 and 9 have been described elsewhere [41]. R_f, MS/EA for all following compounds are reported in Table 3.

4.1.3.1. N-((S)-4-Amino-5-morpholine-4-yl-5-oxo-pentyl)-N′-nitro-guanidine (H2N-Arg(NO2)-A2). This product was obtained from 0.50 g of Boc-Arg(NO_2)-A2 (1.31 mmol) and 10 mL of TFA/CH_2Cl_2 solution. 0.515 g of product as trifluoroacetate salt was isolated (quantitative yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.75 (m, 2H), 1.94 (m, 2H), 3.36 (m, 2H), 3.63 (m, 4H), 3.75 (m, 2H), 3.81 (m, 2H), 4.58 (t, 1H); IR (CH_2Cl_2 liquid film on NaCl) ν_{max} (cm^{-1}): 1684, 1447, 1263, 1103, 1037, 850, 721.

4.1.3.2. N-[(S)-4-Amino-5-(4-ethyl-piperazin-1-yl)-5-oxo-pentyl]-N′-nitro-guanidine (H2N-Arg(NO2)-A4). This product was obtained from 4.70 g of Boc-Arg(NO_2)-A4 (11 mmol) and 20 mL of solution of TFA/CH_2Cl_2 solution. 2.92 g of the product as free amine was isolated (80% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.09 (t, 3H, $J = 7.1$ Hz), 1.43 (s, 9H), 1.65 (m, 7H), 1.76 (m, 8H), 2.43 (m, 6H), 3.28–3.73 (m, 6H), 4.56 (m, 1H), 5.85 (d, 1H, $J = 7.5$ Hz), 7.80 (br, 2H), 8.76 (br, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 10.4, 27.7, 31.20, 41.5, 42.1, 44.4, 52.2, 52.9, 54.2, 162.1, 169.8; IR (CH_2Cl_2 liquid film on NaCl) ν_{max} (cm^{-1}): 1710–1630, 1439, 1294, 1205, 1131.

4.1.3.3. N-[5-(4-Acetyl-piperazin-1-yl)-4-(S)-amino-5-oxo-pentyl]-N′-nitro-guanidine (H2N-Arg(NO2)-A5). This product was obtained from 5.09 g of raw Boc-Arg(NO_2)-A5 (9.12 mmol) and 50 mL of solution of TFA/CH_2Cl_2. The product was isolated as trifluoroacetate white solid (4.1 g, quantitative yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.69 (m, 2H), 1.93 (m, 2H), 2.14 (s, 3H), 3.33 (m, 2H), 3.50–3.71 (m, 8H), 4.58 (t, 1H, $J = 4.5$ Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 20.8, 27.5, 40.4, 41.4, 41.8, 42.4, 42.7, 45.1, 45.3, 45.8, 46.2, 50.8, 159.2, 168.1, 173.1; IR (CH_2Cl_2 liquid film on NaCl) ν_{max} (cm^{-1}): 3400–2900, 1691, 1634, 1518, 1439, 1368, 1267, 1202, 1132, 1000, 835, 800, 722.

4.1.3.4. (12-{4-[2-(S)-Amino-5-(N′-nitro-guanidino)-pentanoyl]-piperazin-1-yl}-12-oxo-dodecyl)-carbamic acid 4-nitro-benzyl ester (H2N-Arg(NO2)-A7). This product was obtained from raw Boc-Arg(NO_2)-A7 (3.67 g) dissolved in TFA/DCM 1:1 (50 mL). The product was isolated as trifluoroacetate salt (3.538 g, quantitative yield). ^{1}H NMR (500 MHz, CD_3OD) δ (ppm): 1.30 (m, 12H), 1.50 (2H, m), 1.61 (m, 2H, $J = 7.3$ Hz), 1.72 (m, 2H), 1.88 (t, 2H, $J = 7.2$), 2.41 (m, 2H), 3.11 (q, 2H, $J = 6.6$ Hz), 3.31 (m, 2H), 3.64 (m, 8H), 4.49 (m, 2H), 5.18 (s, 2H), 7.57 (d, 2H, $J = 8.7$ Hz), 8.20 (d, 2H, $J = 8.7$ Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 26.41, 28.79, 27.78, 30.37, 30.43, 30.50, 30.61, 30.62, 30.84, 34.08, 41.18, 41.86, 42.21, 42.69, 43.18, 43.59, 46.19, 46.54, 46.61, 51.44, 65.87, 124.56, 129.03,

146.39, 148.86, 158.42, 158.42, 168.71, 168.68, 174.47, 177.54.

4.1.3.5. {2-[2-(2-{4-[2(S)-Amino-5-(N′-nitro-guanidino)-pentanoyl]-piperazin-1-yl}-ethoxy)-ethoxy]-ethyl}-carbamic acid 4-nitro-benzyl ester (H_2N-Arg(NO_2)-A8). This product was obtained from 3.38 g of Boc-Arg(NO_2)-A8 (4.08 mmol) and 50 mL of TFA/CH_2Cl_2. The product was isolated as trifluoroacetate salt (4.805 g, quantitative yield). The product was then neutralized for the next reaction step by dissolution in NaOH 1 N and extraction with CH_2Cl_2 off the aqueous phase saturated with NaCl. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.70 (m, 2H), 1.88 (m, 2H), 3.33 (m, 8H), 3.42 (m, 2H), 3.60 (m, 2H), 3.68 (t, 4H), 3.83 (m, 4H), 4.57 (m, 1H), 5.20 (s, 2H), 7.55 (d, 2H, $J = 8.7$ Hz), 8.20 (d, 2H, $J = 8.7$ Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 39.5, 40.4, 40.8, 42.5, 48.9, 50.7, 51.6, 56.6, 63.7, 65.8, 69.6, 69.7, 69.9, 124.0, 127.9, 144.8, 147.3, 158.3, 159.1, 168.2; IR (CH_2Cl_2 liquid film on NaCl) ν_{max} (cm^{-1}): 3328, 2936, 1676, 1608, 1522, 1450, 1349, 1271, 1203, 1132, 836, 800, 740, 722.

4.1.4. General procedure for the preparation of arylsulfonic acid [4-guanidino-1(S)-(azacyclohexan-1-ylcarbonyl) butyl]amide (Sj-Arg(NO_2)-Ai; Scheme 1, step iii).

Product H_2N-Arg(NO_2)-Ai (0.54 mmol, 1 equiv) was dissolved in CH_2Cl_2 (5 mL) and Et_3N (0.169 g, 1.67 mmol, 3.1 equiv). Then arylsulfonyl chloride (0.64 mmol, 1.2 equiv) in CH_2Cl_2 (1 mL) was added dropwise with stirring at 0–5 °C. The solution was stirred for additional 2 h at room temperature, then the mixture was washed with brine, dried over $MgSO_4$, evaporated under reduced pressure and purified by column chromatography on SiO_2 or SFC (supercritical fluid chromatography, Mettler-Toledo facilities).

Compounds S1-Arg(NO_2)-Ai with i being 1, 3, 6 and 9 have been described elsewhere [41]. R_f, MS/EA for all following compounds are reported in Table 3.

4.1.4.1. N-[4-(S)-(N′-Nitro-guanidino)-1-(morpholine-4-carbonyl)-butyl]-3-trifluoromethyl-benzenesulfonamide (S1-Arg(NO_2)-A2). This product was obtained from 0.60 mmol of NH_2-Arg(NO_2)-A2 and 0.60 mmol of 3-trifluoromethyl-benzenesulfonyl chloride. After workup 0.256 g of raw product was obtained and submitted to next reaction step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.64 (m, 2H), 1.84 (m, 2H), 3.30 (m, 8H), 3.54 (m, 2H), 4.14 (m, 1H), 6.65 (d, 1H, $J = 8.6$ Hz), 7.55 (br, 2H), 7.68 (t, 1H, $J = 7.7$ Hz), 7.84 (d, 1H, $J = 7.7$ Hz), 8.05 (d, 1H), 8.07 (s, 1H), 8.65 (br, 1H); IR (CH_2Cl_2 liquid film on NaCl) ν_{max} (cm^{-1}): 3233, 2925, 2859, 1634, 1538, 1429, 1327, 1268, 1165, 1133, 1113, 1071, 1034.

4.1.4.2. N-[1-(4-Ethyl-piperazine-1-carbonyl)-4-(N′-nitro-guanidino)-butyl]-3-trifluoromethyl-benzenesulfonamide (S1-Arg(NO_2)-A4). This product was obtained from 0.60 mmol of NH_2-Arg(NO_2)-A4 and 0.76 mmol of 3-trifluoromethyl-benzenesulfonyl chloride. After workup only traces of product were isolated (yield <10%).

4.1.4.3. (12-{4-[5-(N′-Nitro-guanidino)-2-(S)-(3-trifluoromethyl-benzenesulfonylamino)-pentanoyl]-piperazin-1-yl}-12-oxo-dodecyl)-carbamic acid 4-nitro-benzyl ester (S1-Arg(NO_2)-A7). This product was obtained from 0.154 g (0.21 mmol, 1 equiv) of the precursor NH_2-Arg(NO_2)-A7, 0.063 g of Et_3N (0.64 mmol, 3 equiv) and 0.060 g of 3-trifluoromethyl-benzenesulfonyl chloride (0.24 mmol, 1.2 equiv). After workup the residue was purified by column chromatography to obtain the desired product (0.106 g, yield 67%). IR (CH_2Cl_2 liquid film on NaCl) ν_{max} (cm^{-1}): 1707, 1627, 1522, 1427, 1346, 1326, 1163, 1130; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.26 (m, 14H), 1.51 (m, 4H), 1.59 (m, 4H), 2.29 (m, 2H), 3.10–3.70 (m, 12H), 4.18 (m, 1H), 4.90 (m, 1H), 5.19 (s, 2H), 7.51 (d, 2H, $J = 8.1$ Hz), 7.68 (t, 1H, $J = 8.7$ Hz), 7.82 (d, 1H, $J = 8.7$ Hz), 8.06 (m, 2H), 8.21 (d, 2H, $J = 8.1$ Hz); ^{13}C NMR (125 MHz, CD_3OD) δ (ppm): 25.4, 26.9, 29.5, 29.6, 29.7, 29.8, 30.2, 33.4, 40.5, 41.4, 42.2, 42.3, 42.4, 45.3, 45.4, 46.3, 52.8, 65.2, 123.2 ($J_{CF} = 273$ Hz), 123.8, 124.3 ($J_{CF} = 4.3$ Hz), 128.2, 129.6, 130.2 ($J_{CF} = 4.3$ Hz), 131.0, 140.7, 144.4, 147.5, 156.0, 159.3, 172.7, 177.7.

4.1.4.4. {2-[2-(2-{4-[(S)-5-Guanidino-2-(3-trifluoromethyl-benzenesulfonylamino)-pentanoyl]-piperazin-1-yl}-ethoxy)-ethoxy]-ethyl}-carbamic acid 4-nitrobenzyl ester (S1-Arg(NO_2)-A8). This product was obtained from 0.22 g of NH_2-Arg(NO_2)-A8 (0.37 mmol, 1 equiv), 0.149 g of Et_3N (1.48 mmol, 4 equiv) and 0.108 g of 3-trifluoromethyl-benzenesulfonyl chloride (0.44 mmol, 1.2 equiv). After workup, the residue was purified by flash chromatography (gradient of EtOAc in CH_3CN from 1:9 to 2:8) to obtain 0.1772 g of desired product (yield 60%). ^{1}H NMR (500 MHz, $CDCl_3$, 44 °C) δ (ppm): 1.60 (m, 2H), 1.67 (m, 2H), 2.12 (m, 1H), 2.22 (m, 1H), 2.37 (m, 1H), 2.44 (m, 1H), 3.17 (m, 2H), 3.30 (m, 2H), 3.40 (m, 4H), 3.52 (m, 2H), 3.60 (m, 8H), 4.10 (t, 2H), 5.21 (s, 2H), 5.38 (br, 1H), 6.24 (br, 1H), 7.14 (br, 2H), 7.50 (d, 2H, $J = 8.7$ Hz), 7.65 (t, 1H), 7.82 (d, 1H), 8.02 (d, 1H), 8.05 (s, 1H), 8.20 (d, 2H, $J = 8.7$ Hz); ^{13}C NMR (125 MHz, $CDCl_3$, 40 °C) δ (ppm): 24.28, 30.03, 41.05, 40.23, 42.13, 45.10, 52.28, 52.77, 53.02, 57.26, 65.14, 68.80, 69.78, 70.29, 123.65, 124.20, 128.03, 129.48, 129.98, 131.72, 140.53, 144.04, 147.66, 155.98, 159.49, 168.19; IR (CH_2Cl_2 liquid film on NaCl) ν_{max} (cm^{-1}): 3328, 2936, 1716, 1634, 1608, 1522, 1430, 1347, 1326, 1262, 1165, 1132, 1100.

4.1.5. General procedure for the preparation of arylsulfonic acid [4-guanidino-1(S)-(azacyclohexan-1-ylcarbonyl)butyl] amide (AiSj; Scheme 1, step iv).

Product Sj-Arg(NO_2)-Ai (1.01 mmol, 1 equiv) was dissolved in a 3:1 mixture of EtOH/AcOH (10 mL). The flask was purged with nitrogen and the catalyst Pd/C 10% (0.091 g, 0.086 mmol, 0.085 equiv) was added. The reaction mixture was then stirred at 50 °C for 12 h under hydrogen atmosphere. The catalyst was filtered off and the solution was concentrated under reduced pressure. The solid residue was crystallized from a solution of H_2O/MeOH 1:1 and washed

with Et_2O. After drying under vacuum, the product was isolated as acetate salt (about 90% yield).

Compounds AiSj with i being 1, 3, 6 and 9 have been described elsewhere [41]. R_f, MS/EA for all following compounds are reported in Table 3.

4.1.5.1. N-[1-(S)-(4-Acetyl-piperazine-1-carbonyl)-4-guanidino-butyl]-3-trifluoromethyl-benzenesulfonamide (A2S1). This product was obtained from 0.058 g of product S1-Arg(NO_2)-A2 (0.11 mmol). 0.0481 g was isolated for a final yield of 81%. ^{1}H NMR (500 MHz, CD_3OD) δ (ppm): 1.62 (m, 2H), 1.72 (m, 2H), 2.09 (s, 3/2H), 2.10 (s, 3/2H), 3.18 (m, 2H), 3.10–3.70 (m, 8H), 4.32 (m, 1H), 7.77 (t, 1H, $J = 8.1$ Hz), 7.93 (d, 1H, $J = 8.1$ Hz), 8.10 (d, 1H, $J = 8.1$ Hz), 8.12 (s, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ (ppm): 21.06, 21.12, 26.0, 31.0, 41.8, 42.0, 42.6, 46.6, 45.9, 42.7, 43.1, 46.2, 47.1, 53.9, 125.1, 130.3, 131.3, 132.0, 158.7; IR (CH_2Cl_2 liquid film on NaCl) ν_{max} (cm^{-1}): 3600–3000, 1637, 1431, 1328, 1166, 1134.

4.1.5.2. N-{1-[4-(12-Amino-dodecanoyl)-piperazine-1-carbonyl]-4-guanidino-butyl}-3-trifluoromethyl-benzenesulfonamide (A7S1). This product was obtained from 0.269 g of the sulfonamide precursor S1-Arg(NO_2)-A7 (0.31 mmol, 1 equiv). The product was isolated as acetate salt (0.161 g, 68% yield). IR (KBr), ν_{max} (cm^{-1}): 3417, 3339, 2927, 2855, 1628, 1467, 1432, 1326, 1165, 1130; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.35 (m, 14H), 1.57 (m, 2H), 1.61 (m, 2H), 1.66 (m, 2H), 1.70 (m, 2H), 2.37 (m, 2H), 2.90 (m, 2H), 3.18 (m, 2H), 3.10–3.70 (m, 8H), 4.32 (m, 1H), 7.77 (t, 1H, $J = 8.7$ Hz), 7.93 (d, 1H, $J = 8.7$ Hz), 8.11 (d, 1H), 8.12 (s, 1H); ^{13}C NMR (125 MHz, CD_3OD) δ (ppm): 25.94, 26.38, 28.66, 27.48, 30.23, 30.40, 30.49, 30.53, 30.59, 30.94, 33.92, 33.98, 40.74, 41.70, 42.69, 42.05, 43.16, 46.01, 46.41, 46.58, 53.76, 124.90, 125.10, 130.40, 131.42, 132.09, 132.25, 143.37, 158.77, 170.80, 170.92, 174.34, 174.47.

4.1.5.3. N-[1-(4-{2-[2-(2-Amino-ethoxy)-ethoxy]-ethyl}-piperazine-1-carbonyl)-4-guanidino-butyl]-4-trifluoromethyl-benzenesulfonamide (A8S1). This product was obtained from S1-Arg(NO_2)-A8 (0.483 g, 0.56 mmol, 1 equiv), Pd/C 10% (0.09 g, 0.085 mmol, 0.015 equiv) and a mixture of EtOH/AcOH 1:1 (10 mL). 0.315 g of product was isolated for a final yield of 75%. ^{1}H NMR (500 MHz, CD_3OD) δ (ppm): 1.55–1.75 (m, 4H), 2.09–2.50 (m, 4H), 2.54 (m, 2H, $J = 5.4$ Hz), 3.08 (m, 2H), 3.18 (m, 2H), 3.21 (m, 1H), 3.34 (m, 1H), 3.43 (m, 2H, $J = 5.1$ Hz), 3.61 (m, 2H, $J = 5.4$ Hz), 3.63 (m, 2H), 3.66 (m, 2H), 3.68 (m, 2H, $J = 5.1$ Hz), 4.30 (m, 2H, $J = 4.2$–9.4 Hz), 7.77 (t, 1H), 7.94 (d, 1H), 8.09 (d, 1H), 8.10 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 25.93, 31.02, 40.72, 41.69, 42.74, 46.19, 53.63, 53.88, 54.60, 58.41, 68.48, 69.39, 71.35, 124.81, 125.11, 130.39, 131.46, 132.08, 132.34, 143.32, 158.78, 170.40; IR (CH_2Cl_2 liquid film on NaCl) ν_{max} (cm^{-1}): 1651, 1555, 1407, 1328, 1167.

4.1.6. General procedure for the preparation of the sulfonamide library by parallel synthesis (see Tables 1 and 2)

3 mmol of each amine (4 equiv) and 9 mmol of NEt_3 (12 equiv) were dissolved in 25 mL of CH_2Cl_2. 3 mmol (4 equiv) of 3-trifluoromethyl-benzenesulfonyl chloride (S1) and 3-methylbenzenesulfonyl chloride (S2) were each dissolved in 10 mL of CH_2Cl_2. 1-Naphthalenesulfonyl chloride (S3) was dissolved in 8 mL of CH_2Cl_2 and 0.01 mL of DMF. Quinolinesulfonyl chloride (S4) was dissolved in 20 mL of pyridine (not soluble in other solvents). The Miniblock™ synthesizer was plugged in the Minimapper™ solvent handling workstation, connecting the refrigerating circulator to the metallic jacket of the synthesizer. 5 mL of each amine solution was transferred in the four positions of a row of the synthesizer. Then 2 mL of S1, S2 and S3 solutions were transferred dropwise in the first three columns, and 5 mL of S4 in the last column. The Miniblock™ synthesizer was then unplugged from the Minimapper™ and shaken for 2 h, shutting down the refrigerating system after 1 h. The reaction mixtures were collected in round bottomed tubes by transfer through the filtration manifold and submitted to liquid–liquid extraction on Allexis™ workstation. The organic layer was washed with brine, and then filtered. The formation of the sulfonamides was confirmed by TLC, MS, and NMR (when required). Typical sulfonamides' analysis profile is detailed for series A5. R_f, MS/EA for all following compounds are reported in Table 3.

4.1.6.1. N-[5-(4-Acetyl-piperazin-1-yl)-4-(S)-amino-5-oxo-pentyl]-N′-nitro-guanidine (S1-Arg(NO_2)-A5). This product was obtained from 0.60 mmol of NH_2-Arg(NO_2)-A5 and 0.76 mmol of 3-trifluoromethyl-benzenesulfonyl chloride. After workup 0.126 g of raw product was obtained and submitted to SFC for purification. 0.058 g of pure product was isolated for an overall yield of 18%. ^{1}H NMR (300 MHz, CD_3OD) δ (ppm): 1.59 (m, 4H), 2.02 (s, 3/2H), 2.00 (s, 3/2H), 2.74–3.54 (m, 10H), 4.34 (m, 1H), 4.56 (m, 1H), 7.68 (t, 1H, $J = 7.5$ Hz), 7.83 (d, 1H, $J = 7.5$ Hz), 8.01 (d, 1H), 8.04 (s, 1H).

4.1.6.2. N-[(S)-1-(4-Acetyl-piperazine-1-carbonyl)-4-(N′-nitro-guanidino)-butyl]-3-methyl-benzenesulfonamide (S2-Arg(NO_2)-A5). This product was obtained from 0.60 mmol of NH_2-Arg(NO_2)-A5 and 0.76 mmol of 3-toluenesulfonyl chloride. After workup 0.191 g of raw product was obtained and submitted to SFC for purification. 0.097 g of pure product was isolated for an overall yield of 33%. ^{1}H NMR (300 MHz, CD_3OD) δ (ppm): 1.59 (m, 4H), 2.01 (s, 3H), 2.32 (s, 3H), 2.74–3.54 (m, 10H), 4.15 (m, 1H), 7.34 (m, 1H), 7.58 (m, 1H).

4.1.6.3. Naphthalene-1-sulfonic acid [(S)1-(4-acetyl-piperazine-1-carbonyl)-4-(N′-methyl-guanidino)-butyl]-amide (S3-Arg(NO_2)-A5). This product was obtained from 0.60 mmol of NH_2-Arg(NO_2)-A5 and 0.76 mmol of naphthalene-1-sulfonyl chloride. After workup 0.210 g of raw product was obtained and submitted to SFC for purification. 0.0920 g of pure product was isolated for an overall yield of 30%. ^{1}H

NMR (300 MHz, CD_3OD) δ (ppm): 1.42 (m, 4H), 1.98 (s, 3H), 2.95–3.40 (m, 10H), 4.12 (m, 1H), 7.12–7.61 (m, 3H), 7.91 (d, 1H, $J = 7.5$ Hz), 8.07 (d, 1H, $J = 8.7$ Hz), 8.17 (d, 1H, $J = 7.5$ Hz), 8.62 (d, 1H, $J = 8.1$ Hz).

4.1.6.4. Quinoline-8-sulfonic acid [1-(S)-(4-acetyl-piperazine-1-carbonyl)-4-(N′-nitro-guanidino)-butyl]-amide (S4-Arg(NO2)-A5). This product was obtained from 0.60 mmol of NH_2-Arg(NO_2)-A5 and 0.76 mmol of naphthalene-1-sulfonyl chloride. After workup 0.286 g of raw product was obtained and submitted to SFC for purification. 0.0872 g of pure product was isolated for an overall yield of 28%. ^{1}H NMR (300 MHz, CD_3OD) δ (ppm): 1.56 (m, 4H), 1.99 (s, 3/2H), 2.02 (s, 3/2H), 2.84–3.38 (m, 10H), 4.20 (m, 1H), 7.61–7.70, 8.17, 8.33–8.40, 9.01 (m, 6H).

4.1.7. General procedure for the final deprotection step by parallel synthesis

Basic requirements for combined heating, H_2 fluxing and vigorous stirring for catalyst suspension were not suitable for Miniblock™ synthesizer. We decided to perform hydrogenation in round bottomed cylindrical vials providing individual magnetic stirring. We fixed them on a solid support made with a perforated polystyrene block (25×25 cm^2, 5 mm width) providing 16 positions. The support was used to allow the simultaneous immersion of the reaction vials in a single oil bath for heating. The vials containing the sulfonamides (0.45–0.75 mmol), 0.02 g of Pd/C catalyst, in 5 mL of a 3:1 mixture of EtOH/AcOH were sealed with a septum, purged with nitrogen and filled with H_2. Two cow receivers for distillation were used to connect two H_2 filled balloons to eight tubes, reducing the number of required balloons to ensure H_2 atmosphere saturation in the reaction vials. The reaction mixtures were stirred at 50 °C for 12 h under hydrogen atmosphere. The catalyst was filtered off, and the filtrates, collected in round bottomed flasks, were concentrated under reduced pressure. The solid residues were crystallized from a solution of H_2O/MeOH 1:1 and washed with Et_2O. The solvent was evaporated and the product was isolated as acetate salt (about 90% yield).

Typical deprotected sulfonamides' analysis profile is detailed for series A5. R_f, MS/EA for all following compounds are reported in Table 3.

4.1.7.1. N-[1-(S)-(4-Acetyl-piperazine-1-carbonyl)-4-guanidino-butyl]-3-trifluoromethyl-benzenesulfonamide (A5S1). This product was obtained from 0.058 g of S1-Arg(NO_2)-A5 (0.11 mmol). 0.0481 g of product was isolated for a reaction yield of 81%. ^{1}H NMR (500 MHz, CD_3OD) δ (ppm): 1.62 (m, 2H), 1.72 (m, 2H), 2.09 (s, 3/2H), 2.10 (s, 3/2H), 3.18 (m, 2H), 3.10–3.70 (m, 8H), 4.32 (m, 1H), 7.77 (t, 1H, $J = 8.1$ Hz), 7.93 (d, 1H, $J = 8.1$ Hz), 8.10 (d, 1H, $J = 8.1$ Hz), 8.12 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 21.1, 21.1, 26.0, 31.0, 41.8, 42.0, 42.6, 46.6, 45.9, 42.7, 43.1, 46.2, 47.1, 53.9, 125.1, 130.3, 131.3, 132.0, 158.7; IR (CH_2Cl_2 liquid film on NaCl) ν_{max} (cm^{-1}): 3600–3000, 1637, 1431, 1328, 1166, 1134.

4.1.7.2. N-[1-(S)-(4-Acetyl-piperazine-1-carbonyl)-4-guanidino-butyl]-3-methylbenzenesulfonamide (A5S2). This product was obtained from 0.097 g of S2-Arg(NO_2)-A5. 0.0847 g of product was isolated for a yield of 85%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm): 1.56 (m, 4H), 1.99 (s, 3/2H), 2.00 (s, 3/2H), 2.32 (s, 3H), 3.06–3.53 (m, 8H), 4.10 (m, 1H), 7.33 (m, 2H), 7.56 (m, 2H).

4.1.7.3. Naphthalene-1-sulfonic acid [1-(S)-(4-acetyl-piperazine-1-carbonyl)-4-guanidino-butyl]-amide (A5S3). This product was obtained from 0.092 g of S3-Arg(NO_2)-A5 (0.20 mmol). 0.0319 g of product was isolated for a final yield of 34%. ^{1}H NMR (300 MHz, CD_3OD) δ (ppm): 1.43 (m, 4H), 1.94 (s, 3/2H), 1.95 (s, 3/2H), 2.70–3.40 (m, 10H), 4.10 (m, 1H), 7.46–7.61 (m, 3H), 7.89 (d, 1H, $J = 7.7$ Hz), 8.05 (d, 1H, $J = 7.7$ Hz), 8.14 (d, 1H, $J = 7.7$ Hz), 8.60 (d, 1H, $J = 7.7$ Hz).

4.1.7.4. 1,2,3,4-Tetrahydro-quinoline-8-sulfonic acid [1-(S)-(4-acetyl-piperazine-1-carbonyl)-4-guanidino-butyl]-amide (A5S4). This product was obtained from 0.0872 g (0.21 mmol) of S4-Arg(NO_2)-A5. 0.0839 g of product was isolated for a final yield of 70%. ^{1}H NMR (300 MHz, CD_3OD) δ (ppm): 1.50 (m, 4H), 1.76 (m, 2H), 1.97 (s, 3H), 2.65 (m, 2H), 3.03 (m, 2H), 3.10–3.45 (m, 10H), 4.10 (d, 1H, $J = 5.7$ Hz), 6.40 (m, 1H, $J = 7.7$ Hz), 6.96 (d, 1H, $J = 7.7$ Hz), 7.32 (d, 1H, $J = 7.7$ Hz).

4.2. Biological assays

4.2.1. Enzyme kinetics

Substrate H-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitroanilide dihydrochloride (S2238) was obtained from Chromogenix. Human thrombin was obtained from Hypen BioMed. Enzyme kinetics studies were performed on a Cary 210 spectrophotometer.

The inhibition activities were measured by adapting the protocol developed by Lottenberg et al. [47]: 100 μL of the solution of substrate S2238 (0.1 mM in water) and 100 μL of a water solution of the tested compound (50–200 μM final concentration) were diluted in 2 mL of Tris–Hepes Buffer (Tris 0.01 M, Hepes 0.01 M, NaCl 0.5 M, PEG 6000 0.1% m/v, pH 7.8). Finally 10 μL of water solution of thrombin (10 NIH/mL, 75.2 nM) was added to start the reaction. The appearance of the substrate hydrolysis product (*p*-nitroaniline) was measured at 405 nm as a function of time. From the slope (m) of the linear curve used to fit the plots of V/V_i versus inhibitor concentration (ratio of hydrolysis in the absence and in the presence of inhibitors) we calculated the inhibition constants from the equation $K_i = K_m/m(1/([S] + K_m)$, where K_m is the Michaelis constant of the system as reported by Lottenberg (1.5 μM [47]) and [S] is the substrate concentration. The obtained inhibition constants are shown in Table 2. Two series of independent measurements are reported.

4.2.2. Surface grafting

Native PET track-etched membranes from Cyclopore-Louvain la neuve (thickness 12 μm, density 1.39 g/cm^2, pore size

0.49 μm, 1.45×10^{-6} pores/cm^2) were cut in disks of 13 mm of diameter. The polymer samples (10 disks) were stirred at 60 °C for 1 h into a mixture of acetone (50 mL), pyridine (1.06 mL) and *p*-toluenesulfonyl chloride (2.5 g). The samples were washed successively with acetone (50 mL, 5 min) and water (50 mL, 5 min). The resulted activated samples (hydroxyl functions of chain-ends transformed into tosylates) were individually immersed in 1 mL of a 10^{-3} M solution of inhibitor in a phosphate buffer (PBS, pH 8)/CH_3CN mixture (1:1) and incubated 2 h at 20 °C with shaking. The samples were washed with PBS/CH_3CN (1 mL, 2×10 min), water (1 mL, 2×5 min), 5×10^{-3} M HCl (1 mL, 2×5 min) and water (1 mL, 2×10 min). The resulting inhibitor grafted membranes were finally dried over filter paper and analysed by XPS within 24 h. The blank samples were prepared in the same way but without addition of *p*-toluenesulfonyl chloride in the activation step.

The surface molar fractions were measured by XPS analysis: PET grafted with A6S1: C 1s = 70.34%, F 1s = 1.23% (F/C = 0.0175); PET blank: C 1s = 68.99%, F 1s = 0.83% (F/C = 0.0120); corrected F/C × 100 = 0.55. The concentration of F tagged molecule on the surface was calculated from the corrected F/C ratio. Considering the polymer repeated unit of PET -CO-C_6H_4-CO_2-CH_2CH_2-O- ($C_{10}H_8O_4$) and the derivatized (by grafting of compound A6S1) chain unit -CO-C_6H_4-CO_2-CH_2CH_2-A6S1 ($C_{39}H_{57}F_3N_7O_6S$) we calculated the percentage of derivatized units as follows. For a mixture 98.1% ($C_{10}H_8O_4$) and 1.9% ($C_{39}H_{51}F_3N_7O_6S$): F/C × 100 = [(1.9 × 3)/(98.1 × 10 + 1.9×39)] × 100 = 5.7/1055.1 × 100 = 0.54 (experimental value 0.55). We previously calculated on the basis of PET crystallographic data and simple geometrical considerations [10] an average of 1.72×10^{15} PET monomer units per cm^2 of surface covering 10 atomic layers (the depth analysed by XPS) or about 2850 pmol/cm^2. Thus, 1.9% of derivatized surface monomer units corresponds to about 54 pmol/cm^2 of fixed compound A6S1.

4.2.3. Clot formation

For the evaluation of material hemocompatibility we used a simple test based on the weight measurement of the blood clot formed on given substrates (polymer squares of 4×4 cm fixed on a cylindrical glass support of 2.6 cm of internal diameter) after blood contact and incubation [50]. Human blood was withdrawn from a healthy volunteer, collected in citrated tubes and used within a day. For coating experiments 100 μL of a 10^{-3} M solution of the tested compound was placed on the polymer surface that was then dried overnight. After weighting the samples, 200 μL of human blood was placed on the surface, 20 μL of $CaCl_2$ was added to start coagulation and the samples were incubated statically for 1.5 h at 37 °C in a saturated water atmosphere and 5% CO_2. The samples were then washed with water (3×2 mL), the clot was fixed with formaldehyde (2 mL of a 5% solution in water) and then washed with water (2 mL). The samples were dried in oven (37 °C) until constant weight. The sample weight differences gave the weight of the formed clot. Native polymer coated with heparin (Leo, 5000 I.E./U.I./mL, 100 μL) was used as anti-clotting reference sample.

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