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A Three-Residue Cyclic Scaffold of non-RGD Containing Peptide Analogues as Platelet Aggregation Inhibitors: Design, Synthesis, and Structure–Function Relationships

Abstract: *Antagonists of fibrinogen at the GPIIb/IIIa receptor, which is the most abundant membrane protein on the platelet surface, are under active investigation as potential antithrombotics. The critical interaction between GPIIb/IIIa and fibrinogen can be inhibited by either linear or cyclic RGDS-containing peptides, which have been proved as lead compounds in the design of platelet aggregation inhibitors. In this study we present the design and construction of a new class of cyclic (S,S) non-RGD containing peptide sequences, using two Cys as a structural scaffold for the development of antiaggregatory agents. The (S,S)–CDC– sequence was incorporated as a conformational constraint, in molecules bearing at least one positive charge with the general formula (S,S)XCDCZ, where X = Ac–Arg, Pro–Arg, Pro–Ser–Lys, and Pro–Ser–Arg, and Z = –NH_2 and Arg–NH_2. Investigation of the structure–function relationships was performed on the basis of (a) the local conformation induced by the (S,S)–CDC motif, (b) the distance of the positively (R–C^{ζ} or K–N^{ζ}) and negatively (D–C^{γ}) charged centers, (c) the presence of a second positive or negative charge on the molecule, and (d) the orientation of the basic and acidic side chains defined by the pseudo dihedral angle (Pdo), which is formed by the R–C^{ζ}, R–C^{α}, D–C^{α}, and D–C^{γ} atoms in the case of (S,S)–RCDC and by the K–N^{ζ}, K–C^{α}, D–C^{α}, and D–C^{γ} atoms in the case of (S,S)–KCDC.*
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Keywords: *cyclic non-RGD containing analogues; cystine non-RGD analogues; platelet aggregation inhibitors; cyclization on solid support; disulfide bond formation*

INTRODUCTION

Integrins, a major family of cellular receptors for extracellular matrix proteins such as fibrinogen, fibronectin, thrombospondin, and von Willebrand factor, have been linked to a variety of angiogenic diseases (cancer, diabetic retinopathy, and rheumatoid arthritis), osteoporosis (reduction in bone mass), and

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restenosis (migration and proliferation of smooth muscle cells). It is also well documented now that the integrins are a family of structurally and functionally related adhesion receptors that participate in embryological development ,normal hemostasis, wound healing and platelet aggregation by the binding of adhesive ligands.[1–6]

Many of the integrin receptors recognize the RGD sequence, found in more than one copies, in their target proteins. Disulfide bonds and hydrogen bonding stabilize the bulk of the target protein, but the RGD sequence is believed to be at the tip of a mobile hairpin, which is formed by two extended antiparallel strands with no regular β-structure.[7–10] It has been proposed that different integrins distinguish differences in the conformation and the sequential environment of various RGD sites.[11,12] Studies with conformationally constrained RGD analogue and nonpeptide RGD mimics have been applied to map the integrin-binding sites and develop potential drug candidates.[7,13,14] The structure activity relationships revealed the occurrence of different type turns in the RGD analogues with higher, equal, or lower integrin affinity when compared with the consensus RGD sequence. In particular, emphasis was given to the presence of a well defined type II′ Gly–Asp β-turn as a prerequisite for integrin binding,[15–17] while selectivity of ligands has been correlated with the distances between the Cβ atoms of Arg and Asp residues.[18]

The concept and the design of agents that control thrombosis by modulating platelet aggregation have received significant attention over the past decade. The primary physiological role of the platelet is to copolymerize with fibrinogen and thus aggregate at sites of injury leading to hemostasis (blood clotting).[3,7,19]

The fibrinogen receptor GPIIb/IIIa($\alpha_{IIb}\beta_3$) is the most abundant membrane protein, member of integrins, on the platelet surface, and is mainly responsible for the aggregation phenomenon. On nonstimulated platelets, GPIIb/IIIa is incapable of binding most of its soluble macromolecular ligands, but after exposure of the cells to appropriate agonists, the receptor undergoes a conformational change as a consequence of signal transmission from inside the cell to the extracellular domain of the receptor and is enabled to interact with fibrinogen and other plasma protein ligands. It has been shown that the sequences RGDS (572–575) and RGDF (95–98) of the α-chain of fibrinogen are recognized by the GPIIb/IIIa receptor and contribute to the aggregation event.[17,20,21] Accordingly, antagonists of fibrinogen at the GPIIb/IIIa receptor are under active investigation as potential anticlotting or antithrombotics. The critical interaction between GPIIb/IIIa and fibrinogen can be inhibited by either linear or cyclic RGDS-containing peptides, which have been proved as lead compounds in the design of platelet aggregation inhibitors.

In this study we present the design and construction of a new class of cyclic (S,S) non-RGD containing peptide sequences, utilizing two cysteine residues as a structural scaffold for the development of promising antiaggregatory agents with biomedical potential. The (S,S)–CDC– sequence was incorporated, as a conformational constraint, in molecules bearing at least one positive charge with the general formula (S,S)XCDCZ, where X and Z various amino acids. Although the presence of two opposite charges on the Arg and Asp side chains, respectively, in the RGD sequence, have been considered as a prerequisite for the expression of the biological activity, their role in the recognition process has not been yet well clarified.[16,22] The target of our design is to stabilize the RGD-like motif, inducing a particular orientation of the positively and negatively charged centers. The following derivatives Ac–RGD–NH_2 (**1**), Ac–RGdD–NH_2 (**2**), (S,S)AcRCDC–NH_2 (**3**) (S,S)Ac–RCDCR–NH_2 (**4**), (S,S)PRCDCK–NH_2 (**5**),(S,S)PSKCDCR–NH_2 (**6**), and (S,S)Ac–DCRCD–NH_2 (**7**) were studied. Investigation of the structure–function relationships was performed on the basis of (a) the local conformation induced by the (S,S)–CDC motif, (b) the distance of the positively (R–C^{ζ} or K–N^{ζ}) and negatively (D–C^{γ}) charged centers, (c) the presence of a second positive or negative charge on the molecule, and (4) the orientation of the basic and acidic side chains defined by the preudo-dihedral angle (Pdo), which is formed by the R–C^{ζ}, R–C^{α} , D–C^{α}, and D–C^{γ} atoms in the case of (S,S)–RCDC and by the K–N^{ζ}, K–C^{α}, D–C^{α}, and D–C^{γ} atoms in the case of (S,S)–KCDC (Figure 1).

MATERIALS AND METHODS

Reagents and solvents were used without further purification 2-(1H-Benzotriazol-1-yl)-1,13,3,-tetramethyluronium tetrafluoroborate (TBTU), hydroxybenzotriazol (HOBt), and *t*-butoxy-carbonyl (Boc-) amino acids were purchased from Neosystem Laboratoire (France). Solvents were purchased from Labscan Ltd. (Ireland), while trifluoroacetic acid (TFA) and diisopropylethylamine (DIEA) were from Merck Schuchardt (Germany) and 4-methyl-benzydrylamine resin (MBHA) was from Saxon Biochemicals GMBH (Hannover, FRG).

Synthesis of the Peptides

The synthesis of the cyclic non-RGD analogues was carried out manually by the stepwise solid phase procedure on a MBHA resin following the Boc chemistry.[23,24] Arginine

Table I Yield (%) and Molecular Mass of the Cyclic and Linear Protected Non-RGD Analogues

	Cyclic Non-RGD Analogues			Linear Cys-Protected Non-RGD Analogues		
Peptide	Yield[a] (%)	Expected MW	Found MW	Yield[b] (%)	Expected MW	Found MW
Ac–RGD–NH_2 (1)	—	—	—	66.0	387.39	387.36
Ac–RGdD–NH_2 (2)	—	—	—	58.0	387.39	387.36
(S,S)Ac–RCDC–NH_2 (3)	12.0	535.60	535.75	5.0[c]	679.76	678.59
(S,S)Ac–RCDCR–NH_2 (4)	40.0	690.81	690.68	40.0	835.98	837.56
(S,S)PRCDCK–NH_2 (5)	40.5	717.90	717.87			
(S,S)PSKCDCR–NH_2 (6)	73.7	804.95	804.71	12.4	950.70	949.77
(S,S)Ac–DCRCD–NH_2 (7)	19.4	649.71	649.52	8.0[c]	793.70	794.00

[a] Calculated as (pure cyclic peptide/crude peptide) × 100.

[b] Linear peptide having both Cys side chains protected with the Acm group. The yield was calculated as (pure linear protected peptide/crude peptide) × 100.

[c] A great percentage of the crude product was found with only one Cys side chain protected with the Acm group.

was introduced as N^{α}–t-Boc–L-Arg(Tos)–OH, cysteine as N^{α}–t-Boc–L-Cys–(Acm)–OH, and aspartic acid as N^{α}–t-Boc-L–Asp(OBzl)–OH. All coupling reactions were performed using a molar ratio of amino acid/TBTU/HOBt/DIEA/resin 3/2.9/3/6/1. After completion of the desired sequence the N-terminal amino group of the compounds (**1**)–(**4**) and (**7**) was acetylated using $(AcO)_2O$/Pyridine in a molar ratio $(AcO)_2O$/resin 30/1.

Cyclization of the Peptides on Solid Support (MBHA Resin)

Formation of the intramolecular disulfide bond of the linear peptides was performed on the resin. In order to facilitate cyclization of the peptides, the resin was of low substitution (from 0.2 to 0.5 meq/g). The removal of the Acm group and the disulfide bond formation were performed by one step reaction, using thallium trifluoroacetate $Tl(tfa)_3$ in dimethylformamide (DMF)/anisole 1% in a molar ratio $Tl(tfa)_3$/peptide 1.2/1 at 0 ^{0}C for 100 min. The resin was then successively washed with DMF, dichloromethane (DCM), MeOH, and DCM, and cleaved from the resin with anhydrous hydrogen fluoride at −8 and 0°C for 15 and 90 min, respectively, in the presence of anisole and phenol as scavengers.

Purification of the crude peptides was performed by high performance liquid chromatography (HPLC; preparative reverse phase C18 column) using gradient elution with the following solvents: A,H_2O/0.1%TFA and B,CH_3CN/0.1%TFA.The purity of the peptide was checked by analytical HPLC and the correct molecular mass was confirmed by electrospray–mass spectroscopy (ES-MS). Yields of the cyclic analogues are ranged from ~12 to 73%, while a substantial amount of the linear Cys-protected analogues was also isolated. Data from the synthesis of the reported peptide analogues are summarized in Table I.

Computational Procedure

Energy minimization (EM) and molecular dynamics (MD) simulations were performed, in order to explore the conformational space, using the programs minimize and dynamic of the TINKER[25,26] molecular modeling package, on a Linux PC (2 × 600 MHz, 512 MB RAM). The Verlet algorithm was used for the numerical integration of motion with the time step of 1 fs, and all bond lengths were constrained by applying the SHAKE algorithm. The simulations were carried in vacuum with dielectric constant set to 4 and using the SASA[27] method for the macroscopic representation of solvent. Atom parameters were taken from the AMBER[28] force field. In both EM and MD simulations, a dual cutoff was used for nonbonded interactions: 15 Å for charge–charge interactions and 10 Å for all other types. Initial structures were built with the protein program of TINKER at the extended conformation and minimized with 1000 steps of conjugate gradients, or until the maximum derivative was less than 0.1. The resulted structures were subjected to 2.2 ns of MD at 300 K using the NVT ensemble and 1 fs as integration step. Structures were saved to disk every 1000 steps (1 ps), so every MD trajectory resulted in 2200 stored conformations. The last 2000 were used for analysis. Potential energy and its components were calculated with the program *analyze* of TINKER package. The programs MOLMOL[29] and WHATIF[30] were used for graphical representation and structural analysis. Secondary structures were analyzed with the DSSP[31] algorithm (within WHATIF). Search for β-turn[32] was also performed (a) by the hydrogen bond formation, (b) the φ/ψ angle distribution, and (c) the general geometrical criterion (the distance Cα(i)–Cα(i+3) to be less than 7Å).

Platelet Aggregation Assays

Assessment of functional blockage of GPIIb/IIIa was made using ADP-induced platelet aggregation in human platelet-rich plasma (PRP). PRP was prepared by centrifugation of whole blood of healthy volunteers anticoagulated with ACD, at 160 g for 10 min. The remaining blood specimen were centrifugated at 3000 g for 20 min to prepare platelet-poor plasma (PPP).

Table II Inhibitory Activities of the Non-RGD Analogues

Peptide Analogues	Inhibition of Platelet Aggregation IC_{50} (μM)
Ac–RGD–NH_2 (1)	500
Ac–RGdD–NH_2 (2)	Inactive
(S,S)Ac–RCDC–NH_2 (3)	105
(S,S)Ac–RCDCR–NH_2 (4)	4.3
(S,S)PRCDCK–NH_2 (5)	10.6
(S,S)PSKCDCR–NH_2 (6)	1.6
(S,S)Ac–DCRCD–NH_2 (7)	569.3

Platelet aggregation was determined by the measured change in light transmission (PPP represents 100%) through the PRP (2.5×10^5 platelet/mL) on a Chrono-log Model 500-Ca Lumi-Aggregometer under stirring conditions.

The aggregation was initiated by adding 10 μM ADP, 1 min after the peptide analogues were added in tris-buffered saline (TBS) at pH 7.4.The reaction was then allowed to proceed for at least 3 min. The ability of the peptides to inhibit platelet aggregation was measured and the IC_{50} value was determined as the concentration of the peptide required to produce 50% inhibition of the response to ADP.

RESULTS AND DISCUSSION

The compounds described were evaluated in platelet aggregation assay to assess their ability to inhibit ADP-induced aggregation of human platelets. The results are given as IC_{50} (concentration required for 50% inhibition) and shown in Table II. Ac–RGD–NH_2 (**1**) is one of the simplest proposed RGD analogues that shows inhibitory activity, while substitution of L-Asp by D-Asp in (**1)** resulted to a completely inactive derivative (**2**), as it has been also reported in the literature.[33] These two compounds, used as controls for the biological assays, indicate that the stereochemistry of the Asp side chain is critical for the antiaggregatory event. Replacement of Gly and Ser in the prototype RGDS sequence by cysteines and formation of the disulfide bond (S,S)Ac–RCDC–NH_2 (**3**) provided a rather effective antagonist of the fibrinogen–GPIIb/IIIa recognition (IC_{50} = 105 μM).This finding supports the assumption that conformationally restricted analogues assume an appropriate structure that could decrease platelet adhesion.

Elongation of the analogue (**3**) from the carboxy terminus of the disulfide bond by an Arg (S,S)Ac–RCDCR–NH_2 (**4**), significantly promoted biological activity (IC_{50} = 4.3 μM), being in agreement with previous reports suggesting that the presence of a positive charge in a position adjacent to Ser of the consensus RGDS sequence, enhances platelet aggregation.[34] Replacement of Lys for Arg with simultaneous expansion by Pro[35] from the N-terminus of (**4**) resulted in to a potent antiaggregatory agent (S,S)PRCDCK–NH_2 (**5**).The most active analogue of this series (S,S)PSKCDCR–NH_2 (**6**) incorporates Lys instead of Arg, and Pro–Ser at the N-terminus part of the (S,S)KCDCR–NH_2 sequence. This correlates well with previous findings suggesting that components of snake venoms isolated as potent inhibitors of platelet aggregation and integrin-dependent cell adhesion invariably contain an KGD sequence.[36] Also, the Pro–Ser sequence at the N-terminus of the RGDS pattern is considered as an antiaggregatory promoter. Compound (S,S)Ac–DCRCD–NH_2 (**7**), which instead of the cyclic (S,S)–CDC– sequence includes (S,S)–CRC– and instead of amino acids with basic side chains (Arg or Lys) comprises Asp residues, is a very weak antithrombotic derivative.

Our findings point out that the (S,S)–R(K)CDC– probe is a conformational constraint that induces antagonist activity, while the occurrence of two positive charges (R,K) increases the antiaggregatory properties.[37] In this regard, the (S,S)-CDC- scaffold accommodates the correct orientation of the Arg(Lys) and Asp side chains that inhibits the binding of fibrinogen to the platelet glycoprotein GPIIb/IIIa. Previous studies have shown that enclosure of the RGD or RGDS sequence in cystine-containing disulfide ring analogues enhances antiaggregatory activity.[16,38] The reported results evidence that (S,S)–CDC– is a lead non-RGD(S) cyclic sequence, which incorporated into molecules of the general formula (S,S) XCDCZ (where X = Ac–Arg , Pro–Arg , Pro–Ser–Lys , Pro–Ser–Arg, and Z = $-NH_2$, Arg–NH_2) display antiaggregatory activity. It is concluded that the overall shape and the resident functionalities of this ring system antagonizes the fibrinogen-GPIIb/IIIa ligation, while the opposite is true for the XCRCZ (where X,Z = Asp), which includes acidic residues.

Analysis of the MD trajectories of all the simulated cyclic peptides revealed that the φ and ψ dihedral angles of the residue included between the two cysteines are constrained with values approximately $-75°$ and $-30°$ respectively. Search for β-turn formation in the segments (S,S)–RCDC–, (S,S)–CRCD–, and (S,S)–KCDC– showed the following: (a) hydrogen-bonded interactions were not observed in any frame, (b) the φ and ψ dihedral angle values of the two central residues of the preceding segments do not correspond to any of the classic β-turn structures, and (c) the distance between C$\alpha(i)$–C$\alpha(i + 3)$ was found greater than 7Å. Taking in consideration that not one of the β-turn known criteria is accomplished in these segments, we conclude that they do not intend to form

Table III Conformational Characteristics of the Cyclic Non-RGD Containing Peptide Analogues Assessed by Molecular Dynamics (MD) Simulations

Peptide Analogues	Average Distance (Å) of the Charged Centers[a]	Number of Frames[b] with $-45° < \text{Pdo}^{d} < 45°$	Average Distance (Å) of the Charged Centers[c] Frames with $-45° < \text{Pdo} < 45°$
(S,S)Ac–RCDC–NH_2 (3)	7.31 (0.38)	15	7.06 (0.38)
(S,S)Ac–RCDCR–NH_2 (4)	9.20 (1.85)	41	8.50 (1.55)
(S,S)RPCDCK–NH_2 (5)	9.12 (1.58)	30	7.65 (1.58)
(S,S)PSKCDCR–NH_2 (6)	8.87 (1.59)	38	8.29 (1.58)
(S,S)Ac–DCRCD–NH_2 (7)	9.21 (1.55)	32	8.82 (1.55)

[a] Average distance (standard deviation in parentheses) of the 50 lowest energy frames between the charged centers in the —Xaa—Cys—Asp— sequence, where Xaa is Arg or Lys. The distance is defined between the Arg—C^{ζ} or Lys–N^{ζ}, and Asp–C^{γ}.

[b] Out of the 50 lowest energy.

[c] Average distance (standard deviation in parentheses) of the frames that fall into the 50 lowest energy frames and have $-45° < \text{Pdo} < 45°$.

[d] Pseudodihedral angle formed by the R–C^{ζ} (K–N^{ζ}), R–C^{α} (K–C^{α}), D–C^{α}, D–C^{γ} atoms.

β-turn structures. Therefore, it appears that the occurrence of a β-turn in the peptide backbone is not directly correlated to the biological activity.

Consequently, our next step was to investigate the orientation and the relative positions of the positively and negatively charged side chains. The orientation of the side chains was examined according to the pseudo dihedral angle (Pdo) (Figure 1) formed by the R–C^{ζ} (K–N^{ζ}), R–C^{α}, D–C^{α}, D–C^{γ} atoms, whereas the relative positions of the charged centers were investigated by measuring the distance between R–C^{ζ} (K–N^{ζ}) and D–C^{γ}. Table III presents some of the simulation results used for the conformational analysis of the peptide analogues. A number of 50 frames, out of 2000, which showed the lowest energy were used for this analysis.

As it is shown (Table III) in a great percentage of frames the Pdo adopts values between $-45°$ and $+45°$, which suggests that the side chains of the acidic and basic residues are oriented toward the same side of the peptide backbone. This orientation is probably induced by the (S,S)–CDC– and (S,S)–CRC– scaffolds. The lowest energy structures from molecular dynamics trajectories for peptide analogues **3**–**7**, generated from MOLSCRIPT,[39] are illustrated in Figure 2. An exception constitutes analogue (S,S)Ac–RCDC–NH_2 (**3**) probably due to the absence of elongation from the carboxy terminus of Cys. It seems that elongation of the (S,S)–C–Xaa–C– pattern (where Xaa is D or R) from both sites reduces the mobility of the Arg (Lys) and Asp side chains. The distance between the positively and negatively charged groups for which the Pdo values are ranged from $-45°$ to $+45°$ is approximately 8 Å, indicating the occurrence of a medium strength ionic interaction. These findings could be correlated to the biological activity of the reported analogues on the basis of the proper orientation of the Arg(Lys) and Asp side chains, induced by the (S,S)–C–Xaa–C– scaffold.

In conclusion, evaluation of the biological and computational results of this study leads to the following observations:

1. The (S,S)–CDC– and the (S,S)–CRC– probes induce a topologically stable conformation to the peptides , which however does not fulfill the criteria of a β-turn like structure for the (S,S)–RCDC–, (S,S)–CRCD–, and (S,S)–KCDC– segments. It is more likely that the

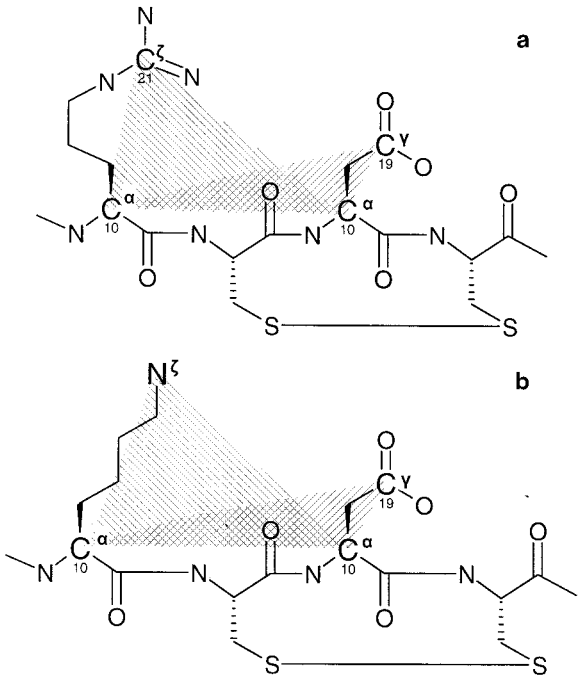

FIGURE 1 Schematic presentation of the pseudo dihedral angle (Pdo) defined (a) by the R–C^{ζ}, R–C^{α}, D–C^{α}, and D–C^{γ} atoms in the sequence (S,S)–RCDC and (b) by the K–N^{ζ}, K–C^{α}, D–C^{α}, and D–C^{γ} atoms in the sequence (S,S)–KCDC.

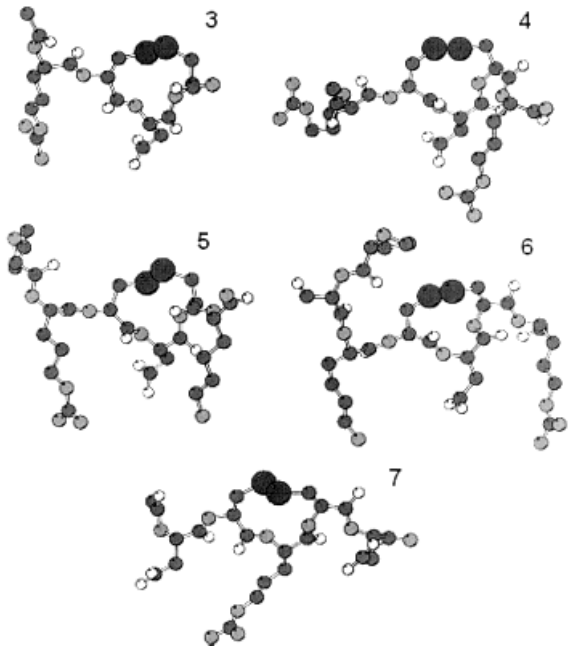

FIGURE 2 The lowest energy structures from molecular dynamics trajectories for peptides **3**–**7**, generated with MOLSCRIPT.[38]

occurrence of a β-turn structure is not a prerequisite for the expression of inhibitory activity against platelet aggregation.

2. The (S,S)–CDC– and (S,S)–CRC– scaffold places the side chains of the basic (Arg or Lys) and the acidic (Asp) residues on the same side of the backbone as evidenced by the values of the pseudo-dihedral angles (Pdo).Our biological results indicate that the orientation of these side chains favors the antiaggregatory reactivity of the designed non-RGD containing peptide analogues.
3. The reported distance between the positive and negative charged centers (~8 Å), which is lower than that usually observed in electrostatic interactions, provides additional evidence for the orientation of the Arg (Lys) and Asp side chains toward the same side of the peptide backbone, confirming also their significance in the biological effect.
4. The low antithrombotic activity of the (S,S)Ac–DCRCD–NH_2 analogue **(7)**, although it displays comparable conformational characteristics with the active derivatives, has to be attributed to the presence of a second negative charge and the absence of a second positive charge in the molecule. In fact, apart of the described constraints induced by the (S,S)–CDC– motif, which favors the antiaggregatory activity, the presence of two positive charges on the side chains (R,K) increases the antiaggregatory properties of the presented analogues.

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