Inhibition of the procoagulant activity of blood platelets by vinylsulfonyl derivatives of pyrrolidine-2-carboxylic acid

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Novel derivatives of 4-(vinylsulfonyl)pyrrolidine-2-carboxylic acids inhibit the exposure of phosphatidylserine by platelets upon activation with thrombin. The heterocyclic inhibitors were synthesized by 1,3-dipolar cycloaddition of divinyl sulfone to azomethine ylides followed by functionalization of the endocyclic nitrogen atom.

Key words: platelets, thrombosis, cysteine proteases, small-molecule inhibitors, vinyl sulfones.

Blood platelets are small (2—5 μm) discoid cell fragments circulating in the blood stream at the concentration of 150—400 thousand μL^{-1}. Platelets are the central element in the hemostasis system, which is responsible for arrest of hemorrhage upon wounds: they can attach to the site of damage and to each other to form a hemostatic plug preventing blood loss. Platelets can also undergo a special process of chaning referred to as "activation" under the action of chemical substances and mechanical action.[1] Upon activation, binding of platelets becomes irreversible; in addition, they secrete clotting factors and expose phosphatidylserine on the outer layer of the cell membrane. Maintenance of the delicate balance between the fluid and non-fluid condition of blood is a non-trivial task for an organism and, therefore, the disfunction of hemostasis system is the leading cause of mortality and disability in the modern world. Among them, thromboses are the most deleterious; they represent formation of blood clots inside vessels, which can result in disturbance of blood supply to body organs. Thromboses occur upon various diseases and conditions, including atherosclerosis, trauma, infarction, stroke, cancer, sepsis, surgical interventions, and so on. For example, up to 70% of the so called sudden cardiac deaths are caused by thromboses,[2] about 400,000 peoples die annualy from this syndrome in the USA alone.[3] Thus, development of novel, efficient and, at the same time, harmless antithrombotic agents is of current concern.

Exposing of phosphatidylserine upon activation, which is generally concentrated in one thrombocytic subpopulation,[4,5] is the most important part of the procoagulant activity of platelets. It leads to the appearance of the binding sites for the proteins of clotting system and dramatic (by several orders of magnitude) acceleration of the reactions of hemostasis system. The studies of the action mechanism of one of the most successful antithrombotic agents, viz., Clopidogrel (the trade name is Plavix) showed the close relationship between the action of the agent and inhibition of the phosphatidylserine-positive subpopulation of platelets.[6] It has been shown earlier that the components of potato tubers inhibiting cysteine proteases can also suppress exposing of phosphatidylserine by platelets.[7] The activity of the inhibitors of cysteine proteases that has been found can be related to their influence on the factor XIIIa, *viz.*, the coagulation cascade transglutaminase whose enzymatic activity is caused by cysteine in the active site.[8] The enzymes containing cysteine in the active site relate to different families, such as proteases, transglutaminases, phosphatases, and the like, depending on the function to be fulfilled. However, from the chemical point of view, the initial step in the reaction of the cysteine fragment with a peptide substrate for the first three families consists in the nucleophilic attack of the sulfhydryl group at the carbonyl carbon atom of the peptide (amide) bond of a substrate to form the activated thioester. Further, depending on the reaction of the activated thioester with an external nucleophile, its hydrolysis (in the case of proteases, the nucleophile is water) or the formation of a new peptide bond (in the case of transglutaminase, the nucleophile is

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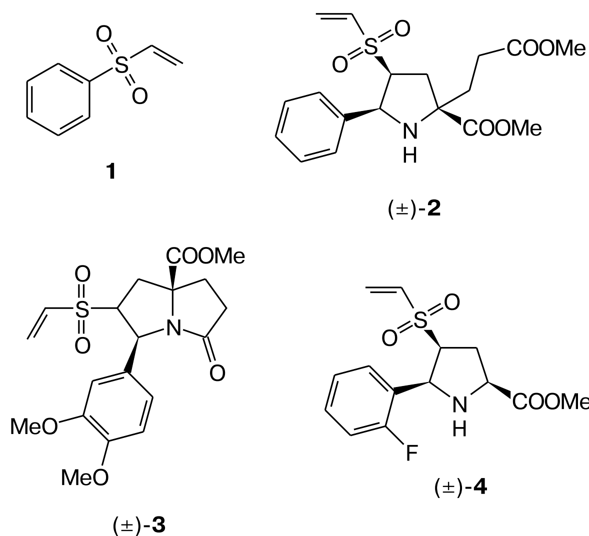
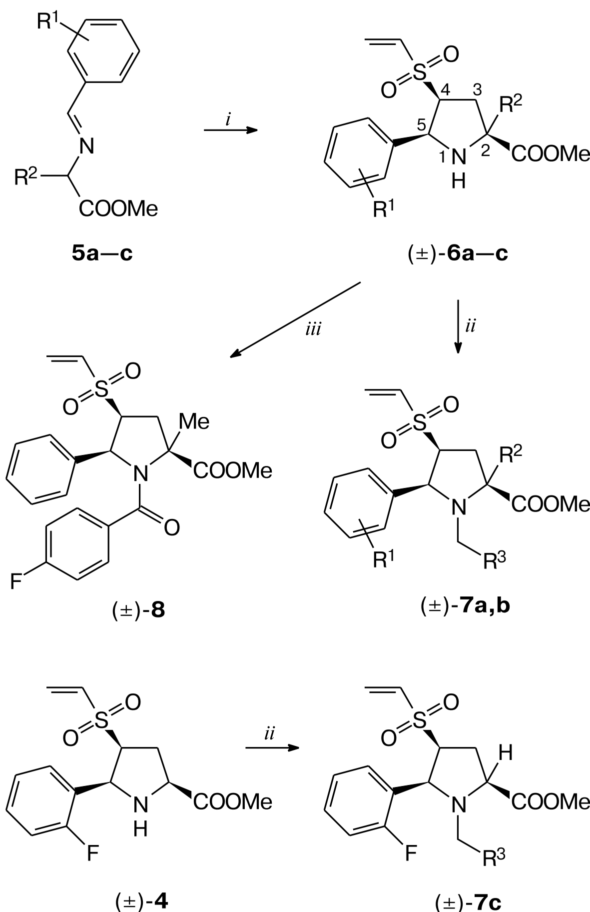
the ω-amino group of lysine; in the case of transpeptidase, the nucleophile is the α-amino group of the terminal amino acid residue) take place. One of the strategies for the inhibition of the enzyme functions is covalent modification of their active sites.[9] The above-mentioned Clopidogrel transforms in the organism to the active metabolite, which inhibits irreversibly the $P2Y_{12}$ purinoceptor by covalent binding to cysteine of the active site.[9] The Michael acceptors[10], in particular, vinyl sulfones,[11] are efficient inhibitors of cysteine proteases. In previous studies, one of the co-authors of the present work has obtained 4-(vinylsulfonyl)pyrrolidine-2-carboxylic acid derivatives and assessed their inhibiting abilities with respect to sortase A of *Staphylococcus aureus*, which is a cysteine transpeptidase.[12,13] Mass spectrometry of the fragment with the established sequences has revealed the covalent modification of the Cys184 amino acid residue of the sortase A active site by one of inhibitors.[13] In the present work, we studied the influence of known[13] and novel pyrrolidinyl vinyl sulfones on exposing of phosphatidylserine by platelets.

In order to establish the specifity of interaction of heterocyclic vinyl sulfones with platelets, we also studied phenyl vinyl sulfone (**1**) as the functional analog. Phenyl vinyl sulfone (**1**) manifests inhibitory properties towards sortase A of *S. aureus*,[14] suppresses adhesion of the *S. aureus* cells to the surface covered by fibronectin,[14] and inactivates some protein tyrosine phosphatases by covalent irreversible modification of cysteine Sγ in the active sites of the enzymes.[15] Pyrrolidinyl vinyl sulfones **2**—**4** inhibit also irreversibly sortase A of *S. aureus*, compound **4** showing the highest activity (see Ref. 13).



To establish the structure-activity relationship of low-molecular-weight 4-(vinylsulfonyl)pyrrolidine-2-carboxylic acid derivatives, we synthesized novel analogs **6**—**8** differing from those obtained earlier in the aryl substituent in the position 5 of the pyrrolidine ring, the substituent R^2 in the position 2, and the substituent at the nitrogen atom (Scheme 1).

Scheme 1



R^1 = 4-Br, R^2 = H (**5a**, **6a**, **7a,b**); R^1 = H, R^2 = Me (**5b**, **6b**);
R^1 = 3-F, R^2 = CH_2Ph (**5c**, **6c**); R^1 = 2-F, R^2 = H (**7c**);
R^3 = Et (**7a**), CH=CHPh (**7b,c**);.

i. Divinyl sulfone, AgOAc, Et_3N, toluene; *ii.* R^3CH_2CHO, $NaBH(OAc)_3$, CH_2Cl_2; *iii.* $4\text{-}FC_6H_4COCl$, THF, Et_3N.

Cycloaddition of divinyl sulfone to the azomethine ylides generated from imino esters **5** under the action of Lewis acids proceeds stereoselectively to form racemic pyrrolidines **6**, wherein the methoxycarbonyl and vinylsulfonyl groups and the 5-aryl substituent are on one side of the pyrrolidine ring (see Scheme 1).[12,16] Modification of the endocyclic nitrogen atom of the pyrrolidine ring was performed by introduction of an alkyl substituent upon reductive amination with propanal and cinnamaldehyde (compounds **7**) and benzoylation with 4-fluorobenzoyl chloride (compound **8**) (see Scheme 1). All the compounds obtained were characterized by ^1H and ^{13}C NMR spectroscopy (the degeneracy of some signals in the ^1H spectra can be due to the fact that one of spin-spin coupling con-

stants is close to zero) and the conclusions on their spatial structures were drawn by comparison of the NMR spectral data with the data of analogs for which the structures were established by X-ray diffraction.[12,16] The purity and composition of pyrrolidinyl vinyl sulfones **6—8** were confirmed by the data from elemental analysis.

Exposing of phosphatidylserine by platelets results in the formation of a population of the so called coated platelets characterizing by a high content of phophatidylserine at the cell surface. These cells bind effectively the fluorescently labeled annexin V, which allows one to quantitate

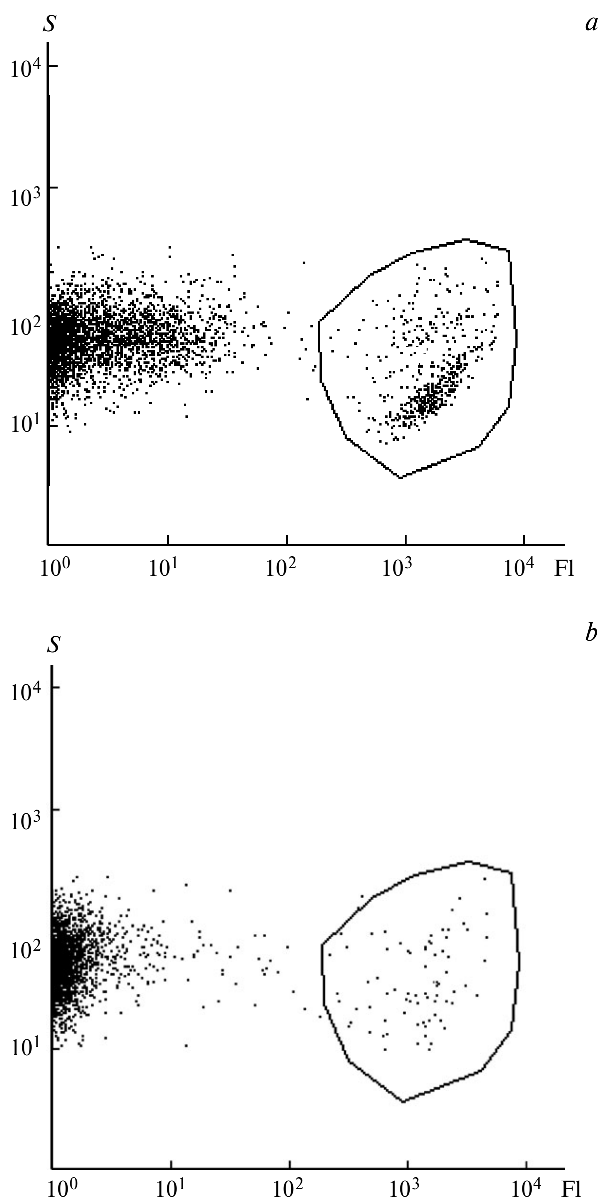


Fig. 1. Flow cytometric study of the formation of the coated platelet population upon activation by thrombin without inhibitors (*a*) and in the presence of vinyl sulfones **7a** (*b*). *S* is the lateral light scattering and *Fl* is the fluorescence of FITC-annexin.

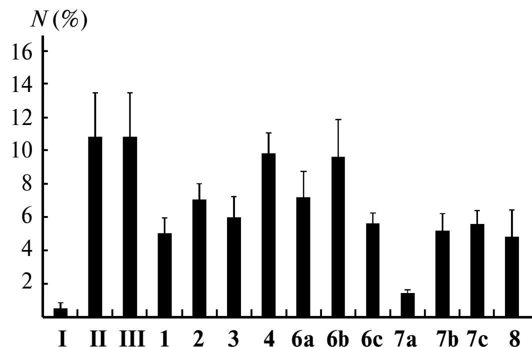


Fig. 2. Influcens of vinyl sulfones on the formation of coated platelets upon activation with thrombin. The mean values ± standard error of the mean (n = 3) are given: I — unactivated platelets; II — control; III — DMSO. *N* is the amount of the annexin-V-positive platelets.

the portion of coated platelets in the population by flow cytometry (Fig. 1). After activation of washed platelets with thrombin, vinyl sulfones **1—4**, **6—8** decreases to different degree the portion of coated platelets (Figs 1 and 2). The inhibitors under study were added at the concentration shown in Table 1. Pyrrolidinyl vinyl sulfone **7a** has the maximum effect, the amount of coated platelets decreased tenfold compared to the control (see Fig. 2). The addition of this compound levels off almost completely the activation effect of thrombin on platelets. The biological activity of the vinyl sulfones under study is not related or related weakly to inhibition of thrombin, which was shown by the study of thrombin inhibition of hydrolysis of a specific substrate in the presence of compounds **6** and **7** (see Table 1).

Table 1. Concentrations of compounds **1—4** and **6—8** upon testing and data for thrombin inhibition in a buffer solution

Compound	*M*	*c*		*I* (%)
		mmol L^{-1}	mg mL^{-1}	
1	168.22	0.594	0.100	15.2
2	381.45	0.163	0.062	9.1
3	409.46	0.049	0.020	−3.1
4	313.35	0.172	0.054	−3.9
6a	374.26	0.096	0.036	−10
6b	309.39	0.149	0.046	−0.9
6c	403.48	0.099	0.040	−1.3
7a	416.34	0.086	0.036	−6.5
7b	490.42	0.159	0.078	20.4
7c	429.51	0.112	0.048	7.5
8	431.49	0.102	0.044	—

Note: *M* is the molecular weight of the compound; *c* is the final concentration of the compound in the test; *I* is thrombin inhibition in a buffer solution at the given concentration of the compound.

Despite a small set of the compounds under study (10 hetetocyclic vinyl sulfones and commercially available phenyl vinyl sulfone), one can draw a preliminary conclusion on the structure—activity relationship for this class of active substances. First, all compounds containing the vinylsulfonyl fragment have an effect on exposing of phosphatidylserine by platelets and it is this structural fragment that causes this activity. Secondly, the heterocyclic vinyl sulfones exhibit a comparable or lowered activity compared to phenyl vinyl sulfone (**1**) except for compound **7a** (see Fig. 2). Thus, the presence of a small (in terms of bulk) alkyl substituent at the endocyclic nitrogen atom appears to be critical for inhibition of platelet activation. We studied also the dependence of the biological activity on the inhibitor concentration for the substituted 4-(vinylsulfonyl)pyrrolidine-2-carboxylates **7a**, **7c**, and **8** (Fig. 3).

The discovery of a novel class of efficient inhibitors of the platelet procoagulant activity acting in the micromolar concentration range (IC_{50} = 20 μmol L^{-1} and IC_{100} = = 90 μmol L^{-1} for pyrrolidinyl vinyl sulfone **7a**) seems to be the most interesting result of the present work for further practical applications. Suppression of the expression of phosphatidylserine by platelets is a promising trend in the prevention and treatment of thromboses, which is confirmed by the data from the study of the drugs being the $P2Y_{12}$ receptor antagonist, such as Clopidogrel, Prasugrel, and Cangrelor.[17] Note that our testing experiments were performed in a buffer solution imitating blood plasma by a number of indices, including the presence of albumin. Thus, one can assume that the same effects at the analogous concentrations of vinyl sulfones will appear in the whole blood.

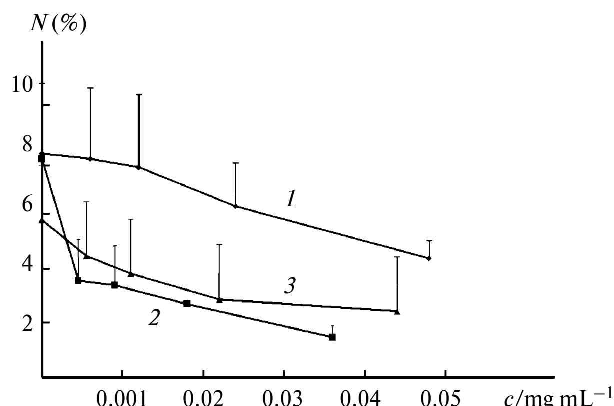


Fig. 3. Dose-dependent decrease in the amount of coated platelets in the presence of inhibitors **7c** (*1*), **7a** (*2*), and **8** (*3*). *N* is the amount of the annexin-V-positive platelets, *c* is the concentration of vinyl sulfones. The mean values ± standard error of the mean (n = 3) are given. The IC_{50} values for these compounds were obtained by the non-linear least-squares method on the assumption of a hyperbolic dependence of inhibition and are 0.0487±0.007 mg mL^{-1} for vinyl sulfone **7c**, 0.0094±0.00028 mg mL^{-1} for vinyl sulfone **7a**, and 0.028±0.005 mg mL^{-1} for vinyl sulfone **8**.

Experimental

Synthetic studies

Divinyl sulfone, aromatic aldehydes, silver(I) acetate, 4-fluorobenzoyl chloride, cinnamaldehyde, and silica gel (Lancaster) were used in the present work. The course of the reactions and the purity of compounds were monitored by TLC using Sorbfil PTLC-AF-A-UV TLC plates in a $CHCl_3$—MeOH (10 : 1) solvent system. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 (400 MHz for ^{1}H and 100 MHz for ^{13}C) instrument at 303 K in DMSO-d_6 or $CDCl_3$ using the residual signals of deuterated solvents as the internal standards.

The syntheses of pyrrolidinyl vinyl sulfones **2**—**4** were described earlier.[12,13,16] Pyrrolidinyl vinyl sulfones **6** were prepared according to the procedure as described for the synthesis of compound **2** (see Ref. 16).

(2*S,4*S**,5*S**)-Methyl 5-(4-bromophenyl)-4-(vinylsulfonyl)-pyrrolidine-2-carboxylate (6a)** was obtained from divinyl sulfone and imino ester **5a**. The yield was 24%. Colorless crystals, m.p. 158—159 °C. ^{1}H NMR (DMSO-d_6), δ: 2.37 (d.d.d, 1 H, H(3), J = 14.0, J = 8.0 Hz, J = 6.6 Hz); 2.51—2.57 (m, 1 H, H(3)); 3.50 (t, 1 H, NH, J = 7.7 Hz); 3.70 (s, 3 H, $COOCH_3$); 3.90 (q, 1 H, H(2), J = 8.2 Hz); 4.05—4.10 (m, 1 H, H(4)); 4.52 (t, 1 H, H(5), J = 7.5 Hz); 5.74 (d, 1 H, $C\underline{H}_2$=CH, J = 16.5 Hz); 5.85 (d, 1 H, $C\underline{H}_2$=CH, J = 9.9 Hz); 6.28 (d.d, 1 H, CH_2=C$\underline{H}$, J = 16.5 Hz, J = 9.9 Hz); 7.38 (d, 2 H_{arom}, J = 8.6 Hz); 7.48 (d, 2 H_{arom}, J = 8.6 Hz). ^{13}C NMR (DMSO-d_6), δ: 30.36, 52.40, 57.72, 62.42, 65.05, 120.91, 129.15, 130.75 (2 C), 131.15 (2 C), 136.50, 137.97, 173.12. Found (%): C, 45.18; H, 4.35; N, 3.63. $C_{14}H_{16}BrNO_4S$. Calculated (%): C, 44.93; H, 4.31; N, 3.74.

(2*S,4*S**,5*S**)-Methyl 2-methyl-5-phenyl-4-(vinylsulfonyl)-pyrrolidine-2-carboxylate (6b)** was obtained from divinyl sulfone and imino ester **5b**. The yield was 73%. Colorless crystals, m.p. 73—75 °C. ^{1}H NMR ($CDCl_3$), δ: 1.56 (s, 3 H, CH_3); 2.33 (d.d, 1 H, H(3), J = 14.8 Hz, J = 8.2 Hz); 3.13 (d.d, 1 H, H(3), J = 14.8 Hz, J = 5.0 Hz); 3.78 (d.d.d, 1 H, H(4), J = 8.2 Hz, J = 6.3 Hz, J = 5.0 Hz); 3.85 (s, 3 H, $COOCH_3$); 4.74 (d, 1 H, H(5), J = 6.3 Hz); 5.38 (d.d, 1 H, CH_2=C$\underline{H}$, J = 16.4 Hz, J = 10.0 Hz); 5.59 (d, 1 H, $C\underline{H}_2$=CH, J = 10.0 Hz); 5.90 (d, 1 H, $C\underline{H}_2$=CH, J = 16.4 Hz); 7.30—7.38 (m, 3 H_{arom}); 7.40—7.43 (m, 2 H_{arom}). ^{13}C NMR ($CDCl_3$), δ: 27.46, 37.69, 52.83, 62.95, 64.35, 68.18, 128.17 (2 C), 128.37 (3 C), 129.45, 134.89, 136.22, 175.29. Found (%): C, 58.38; H, 6.27; N, 4.66. $C_{15}H_{19}NO_4S$. Calculated (%): C, 58.23; H, 6.19; N, 4.53.

(2*S,4*S**,5*S**)-Methyl 2-benzyl-5-(3-fluorophenyl)-4-(vinylsulfonyl)pyrrolidine-2-carboxylate (6c)** was obtained from divinyl sulfone and imino ester **5c**. The yield was 64%. Oily substance. ^{1}H NMR (DMSO-d_6), δ: 2.39 (d.d, 1 H, H(3), J = 14.4 Hz, J = 8.3 Hz); 2.75 (d.d, 1 H, H(3), J = 14.4 Hz, J = 6.0 Hz); 3.06 (s, 2 H, CH_2); 3.40 (d, 1 H, NH, J = 8.5 Hz); 3.67 (s, 3 H, $COOCH_3$); 4.16 (q, 1 H, H(4), J = 6.9 Hz); 4.82 (t, 1 H, H(5), J = 7.6 Hz); 5.72 (d, 1 H, $C\underline{H}_2$=CH, J = 16.5 Hz); 5.82 (d, 1 H, $C\underline{H}_2$=CH, J = 9.9 Hz); 6.24 (d.d, 1 H, CH_2=C$\underline{H}$, J = 16.5 Hz, J = 9.9 Hz); 7.06—7.15 (m, 2 H_{arom}); 7.18—7.36 (m, 7 H_{arom}). ^{13}C NMR (DMSO-d_6), δ: 34.99, 44.37, 52.46, 61.23, 65.47, 68.75, 114.72 (d, J = 21.0 Hz), 115.72 (d, J = 22.0 Hz), 124.91,

127.16, 128.60 (2 C), 129.07, 129.85 (d, J = 8.0 Hz), 130.27 (2 C), 136.37, 137.11, 141.46 (d, J = 6.6 Hz), 162.20 (d, J = 242.0 Hz), 174.11. Found (%): C, 62.35; H, 5.37; N, 3.69. $C_{21}H_{22}FNO_4S$. Calculated (%): C, 62.52; H, 5.50; N, 3.47.

Reductive amination of pyrrolidinyl vinyl sulfones 6 (general procedure). To a solution of pyrrolidinyl vinyl sulfone **6** (1.5 mmol) in CH_2Cl_2 (20 mL), an aldehyde (1.65 mmol) was added. Then $NaBH(OAc)_3$ (0.444 g, 2.10 mmol) was added portionwise with stirring. The reaction mixture was stirred at room temperature for 8 h and a saturated solution of $NaHCO_3$ (10 mL) was added. The organic phase was separated and the aqueous phase was extracted with CH_2Cl_2 (2×10 mL). The combined organic phase was dried with Na_2SO_4, filtered and the filtrate was concentrated *in vacuo*. The residue was chromatographed on silica gel using $CHCl_3$ and $CHCl_3$—MeOH (10 : 1) as an eluent.

(2*S,4*S**,5*S**)-Methyl 5-(4-bromophenyl)-1-propyl-4-(vinylsulfonyl)pyrrolidine-2-carboxylate (7a)** was obtained according to the general procedure from vinyl sulfone **6a** and propanal. The yield was 53%. Colorless crystals, m.p. 102—103 °C. ^{1}H NMR (DMSO-d$_6$), δ: 0.64 (t, 3 H, CH_3, J = 7.3 Hz); 1.06—1.25 (m, 2 H, CH_2); 2.28—2.47 (m, 3 H, CH_2N, H(3)); 2.54—2.61 (m, 1 H, H(3)); 3.55 (d.d, 1 H, H(4), J = 9.6 Hz, J = 6.8 Hz); 3.70 (s, 3 H, $COOCH_3$); 4.18—4.23 (m, 2 H, H(5), H(2)); 5.82 (d, 1 H, C$\underline{H}_2$=CH, J = 16.4 Hz); 5.94 (d, 1 H, C$\underline{H}_2$=CH, J = 9.9 Hz); 6.28 (d.d, 1 H, CH_2=C$\underline{H}$, J = 16.4 Hz, J = 9.9 Hz); 7.44—7.48 (m, 4 H$_{arom}$). ^{13}C NMR (DMSO-d$_6$), δ: 11.94, 20.81, 30.65, 52.43, 56.11, 64.20, 64.36, 66.84, 121.19, 130.10, 130.62 (2 C), 132.27 (2 C), 136.19, 139.49, 173.44. Found (%): C, 49.16; H, 5.34; N, 3.53. $C_{17}H_{22}BrNO_4S$. Calculated (%): C, 49.04; H, 5.33; N, 3.36.

(2*S,4*S**,5*S**)-Methyl 5-(4-bromophenyl)-1-cinnamyl-4-(vinylsulfonyl)pyrrolidine-2-carboxylate (7b)** was obtained according to the general procedure from vinyl sulfone **6a** and cinnamaldehyde. The yield was 90%. Oily substance. ^{1}H NMR (CDCl$_3$), δ: 2.56 (t, 2 H, C$\underline{H}_2$CH=CH, J = 8.5 Hz); 3.30 (d.d, 1 H, H(3), J = 14.4 Hz, J = 8.0 Hz); 3.54 (d.d, 1 H, H(3), J = 14.4 Hz, J = 5.8 Hz); 3.64 (t, 1 H, H(2), J = 8.0 Hz); 3.70 (s, 3 H, $COOCH_3$); 3.82 (q, 1 H, H(4), J = 8.8 Hz); 4.32 (d, 1 H, H(5), J = 8.8 Hz); 5.61 (d.d, 1 H, CH_2=C$\underline{H}$, J = 16.4 Hz, J = 9.9 Hz); 5.82 (d, 1 H, C$\underline{H}_2$=CH, J = 9.9 Hz); 6.02 (d, 1 H, C$\underline{H}_2$=CH, J = 16.4 Hz); 6.06—6.13 (m, 1 H, CH_2C$\underline{H}$ = CH); 6.40 (d, 1 H, CH_2CH=C$\underline{H}$, J = 15.9 Hz); 7.24—7.32 (m, 5 H$_{arom}$); 7.49—7.54 (m, 4 H$_{arom}$). ^{13}C NMR (CDCl$_3$), δ: 30.41, 52.32, 54.16, 62.92, 65.99, 66.11, 122.46, 124.06, 126.35 (2 C), 127.81, 128.59 (2 C), 130.81, 131.19 (2 C), 131.87 (2 C), 134.15, 134.35, 136.42, 136.65, 172.78. Found (%): C, 56.56; H, 5.07; N, 2.58. $C_{23}H_{24}BrNO_4S$. Calculated (%): C, 56.33; H, 4.93; N, 2.86.

(2*S,4*S**,5*S**)-Methyl 1-cinnamyl-5-(2-fluorophenyl)-4-(vinylsulfonyl)pyrrolidine-2-carboxylate (7c)** was obtained according to the general procedure from vinyl sulfone **4** and cinnamaldehyde. The yield was 71%. Colorless crystals, m.p. 122—123 °C. ^{1}H NMR (DMSO-d$_6$), δ: 2.38 (d.t, 1 H, H(3), J = 12.9 Hz, J = 8.8 Hz); 2.51—2.57 (m, 1 H, H(3)); 3.30 (d.d, 1 H, C$\underline{H}_2$CH=CH, J = 14.5 Hz, J = 8.0 Hz); 3.39—3.44 (m, 1 H, C$\underline{H}_2$CH=CH); 3.57 (s, 3 H, $COOCH_3$); 3.62 (d.d, 1 H, H(2), J = 8.8 Hz, J = 7.3 Hz); 4.19 (q, 1 H, H(4), J = 8.6 Hz); 4.53 (d, 1 H, H(5), J = 8.8 Hz); 5.76 (d, 1 H, C$\underline{H}_2$=CH, J = 16.4 Hz); 5.93 (d, 1 H, C$\underline{H}_2$=CH, J = 9.9 Hz); 6.14 (d.d.d, 1 H, CH_2C$\underline{H}$=CH, J = 16.0 Hz, J = 8.0 Hz, J = 6.3 Hz); 6.34 (d, 1 H, CH_2CH=C$\underline{H}$, J = 16.0 Hz); 6.40 (d.d, 1 H, CH_2=C$\underline{H}$, J = 16.4 Hz, J = 9.9 Hz); 7.01—7.06 (m, 1 H$_{arom}$); 7.17—7.24 (m, 2 H$_{arom}$); 7.25—7.33 (m, 5 H$_{arom}$); 7.71—7.75 (m, 1 H$_{arom}$). ^{13}C NMR (DMSO-d$_6$), δ: 30.40, 52.34, 54.22, 59.30, 63.05 (2 C), 114.97 (d, J = 21.0 Hz), 123.97, 124.85 (d, J = 12.0 Hz), 125.53, 126.62 (2 C), 128.03, 128.99 (2 C), 130.00 (d, J = 8.0 Hz), 130.32, 131.82, 133.25, 136.11, 136.83, 161.48 (d, J = 245.0 Hz), 173.03. Found (%): C, 64.45; H, 5.57; N, 3.55. $C_{23}H_{24}FNO_4S$. Calculated (%): C, 64.32; H, 5.63; N, 3.26.

(2*S,4*S**,5*S**)-Methyl 1-(4-fluorobenzoyl)-2-methyl-5-phenyl-4-(vinylsulfonyl)pyrrolidine-2-carboxylate (8).** Vinyl sulfone **6b** (0.272 g, 0.88 mmol) was dissolved in THF (20 mL) and Et_3N (0.135 mL, 0.098 g, 0.97 mmol) was added. 4-Fluorobenzoyl chloride (0.117 mL, 0.154 g, 0.97 mmol) was added dropwise with stirring. The reaction mixture was stirred at room temperature for 12 h and then a saturated solution of NH_4Cl (20 mL) was added. CH_2Cl_2 (100 mL) was added after 10 min and the organic phase was separated and dried with Na_2SO_4. The filtrate was concentrated *in vacuo*. The residue was chromatographed on silica gel using hexane—AcOEt (2 : 1) as an eluent to obtain colorless oily substance (0.352 g) crystallized on storage in a freezer. The yield was 93%. Colorless crystals, m.p. 167—168 °C. ^{1}H NMR (DMSO-d$_6$), δ: 1.69 (s, 3 H, CH_3); 2.31 (d.d, 1 H, H(3), J = 12.3 Hz, J = 6.2 Hz); 2.62 (t, 1 H, H(3), J = 13.3 Hz); 3.80 (s, 3 H, $COOCH_3$); 4.73 (d.d.d, 1 H, H(4), J = 14.2 Hz, J = 8.2 Hz, J = 6.2 Hz); 5.41 (d, 1 H, H(5), J = 8.2 Hz); 5.74—5.80 (m, 1 H, CH_2=C$\underline{H}$); 5.88—5.93 (m, 2 H, C$\underline{H}_2$=CH); 6.95—7.05 (m, 4 H$_{arom}$); 7.10—7.17 (m, 3 H$_{arom}$); 7.29—7.32 (m, 2 H$_{arom}$). ^{13}C NMR (DMSO-d$_6$), δ: 20.89, 36.82, 53.04, 62.80, 64.74, 65.68, 115.11, 115.33, 127.85 (2 C), 128.37, 128.47, 128.56, 129.68 (2 C), 131.72, 133.34, 134.65, 137.61, 161.22, 168.07, 172.98. Found (%): C, 61.48; H, 5.18; N, 3.51. $C_{22}H_{22}FNO_5S$. Calculated (%): C, 61.24; H, 5.14; N, 3.25.

Biochemical studies

Materials. Phenyl vinyl sulfone (Lancaster, United Kingdom), thrombin (Haemotologic Technologies, USA); prostaglandin E1 (MP Biochemicals, USA), and fluorescein (FITC)-conjugated annexin V (Southern Bioech, USA) were used in the work. The remaining reagents for biochemical studies were obtained from Sigma (USA).

Isolation of platelets. Platelets were obtained from the blood of healthy donors prepared in a 3.8% solution of sodium citrate, pH 5.5. The blood:citrate ratio was 9 : 1. To prevent activation of platelets, prostanglandin E1 (1 μmol L^{-1}) and apyrase (0.1 u mL^{-1}) were added to blood. This was centrifuged at 100 *g* at room temperature for 8 min. After centrifugation, the layer enriched in plasma platelets was withdrawn and a 3.8% solution of sodium citrate (pH 5.5) was added to prevent cell aggregation (the plasma : citrate ratio is 3 : 1). Platelets were concentrated by centrifugation at 400 *g* for 5 min at room temperature, resuspended in buffer A (300 μL) (20 *mM* HEPES, 150 *mM* NaCl, 2.7 *mM* KCl, 1 *mM* $MgCl_2$, 0.4 *mM* NaH_2PO_4, 5 *mM* glucose, 0.5% bovine serum albumin, pH 7.4), and finally purified from plasma proteins by gel chromatography on a column with Sepharose CL-2B and equilibrated with buffer A.

Determination of the influence of inhibitors on the formation of platelet heterogenicity upon activation. Following gel filtration, the platelets at the concentration of 20 thousand μL^{-1} were activated for 15 min at room temperature in the absence (control) or in the presence of inhibitors. The concentration of inhibitors

was chosen such that the final concentration of DMSO was 0.2%. Cell were activated by adding 100 *nM* thrombin in buffer A in the presence of 2.5 *mM* $CaCl_2$ and 0.2% fluorescently labeled annexin V. The degree of activation was assessed by the amount of cells binding the fluorescently labeled annexin V on FACS-Calibur (BD Biosciences) flow cytometer using the WinMDI 2.8 (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA) program.

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