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Conformationally tailored *N*-[(2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl)carbonyl]proline templates as molecular tools for the design of peptidomimetics. Design and synthesis of fibrinogen receptor antagonists †

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The proline peptide bond was shown by 2D proton NMR studies to exist exclusively in the *trans* conformation in benzyl (2*S*)-1-{[(2*S*)-2-methyl-6-nitro-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl]carbonyl}-2-pyrrolidinecarboxylate [(*S,S*)-**11**], benzyl (2*S*)-1-{[(2*S*)-2-methyl-7-nitro-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl]carbonyl}-2-pyrrolidinecarboxylate [(*S,S*)-**9**], and in the corresponding 6-amino and 7-amino carboxylic acids (*S,S*)-**3** and (*S,S*)-**4**. On the other hand, the diastereomers (*R,S*)-**11** and (*R,S*)-**9** containing an (*R*) [2-methyl-6/7-nitro-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl]carbonyl moiety, and the diastereoisomers (*R,S*)-**3** and (*R,S*)-**4** incorporating an (*R*) [6/7-amino-2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl]carbonyl moiety were found to exist as equilibria of *trans* (63–83%) and *cis* (17–37%) isomers. These conformationally defined templates were applied in the construction of RGD mimetics possessing antagonistic activity at the platelet fibrinogen receptor.

Introduction

Bioactive peptides bind to biological macromolecules in specific conformations. The exploration of a binding conformation of inherently flexible bioactive peptides is a crucial step in attempts to obtain potent and selective peptidomimetic therapeutic agents. Restriction of the degrees of conformational freedom of native peptides by designing conformationally constrained analogues which imitate the bioactive conformation of the peptide is an important method for the development of peptidomimetics.[1–4]

In the field of peptidomimetic drug design a large number of approaches that reduce the flexibility, and thus the number of conformations accessible to a peptide molecule, have been described.[2,4] Among them, proline or proline mimetics with different ring sizes have frequently been introduced into the sequence of bioactive peptides in order to induce local constraints.[4–7] Proline restricts the local conformational freedom by locking the Φ angle at about $-60°$ due to restricted rotation about the N^{α}–C^{α} bond, included in the pyrrolidine ring. Additionally, proline exhibits hindered rotation around the C^{α}–C(O) bond due to the steric interactions of the pyrrolidine ring and the carbonyl group. As a result of these features, proline is able to affect the conformation of the preceding residue. The most interesting conformational characteristic of proline in peptides is its potential for *cis*/*trans* isomerism of the X-Pro peptide bond, in contrast to other amino acids which form predominantly *trans* peptide bonds.[2,4]

Recently, we have shown that the *cis*/*trans* ratio of the proline peptide bond can be strongly influenced by the chirality of the acyl residue preceding proline.[8] Moreover, we demonstrated that the acyl moiety derived from (*S*)-2,6-dimethyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-2-carboxylic acid [(*S*)-**1**] in acyl-Pro molecules influences the isomerization of the proline peptide bond, constraining the ω dihedral angle to the *trans* orientation. For this reason, we considered *N*-[(2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl)-2-carbonyl]-(*S*)-proline templates as promising, conformationally defined, chiral peptidomimetic building blocks[8] that could be applied, *inter alia,* for the design of fibrinogen receptor antagonists.

The fibrinogen receptor is a member of the integrin family of proteins which are heterodimeric transmembrane glycoproteins. Integrin receptors play an important role in, *for example,* platelet aggregation,[9–11] angiogenesis,[10,12] cell growth and differentiation,[10,13] tumour promotion,[9,12,13] cell adhesion,[9,10,14] bone resorption[9,10,15,16] and immunological processes.[10,17] Antagonists of the fibrinogen receptor, possessing anti-aggregatory activity, are generally mimetics of the Arg-Gly-Asp (RGD) sequence in which the distance between the anionic and cationic centres, which ranges from 13 to 16 Å, is crucial for binding to the receptor.[18–20]

We hypothesized that, in a manner similar to (2*S*)-1-[(2*S*)-2,6-dimethyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl)carbonyl]-2-pyrrolidinecarboxylic acid [(*S,S*)-**2**], which exhibits an exclusively *trans* proline amide bond, and (2*S*)-1-[(2*R*)-2,6-dimethyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl)-carbonyl]-2-pyrrolidinecarboxylic acid [(*R,S*)-**2**], which assumes an equilibrium of 72% *trans* and 28% *cis* conformers in dimethylsulfoxide solution, 1-[(6-amino-2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl)carbonyl]-2-pyrrolidinecarboxylic acid [(*S,S*)-**3**] and its 7-amino isomer (*S,S*)-**4** also should possess *trans* proline bonds, while simultaneously allowing easy functionalization at the aromatic ring (Fig. 1). Based on this assumption, conformationally defined diastereomers of templates **3** and **4** emerged as promising peptidomimetic

† Electronic supplementary information (ESI) available: experimental details. See http://www.rsc.org/suppdata/ob/b4/b400490f/

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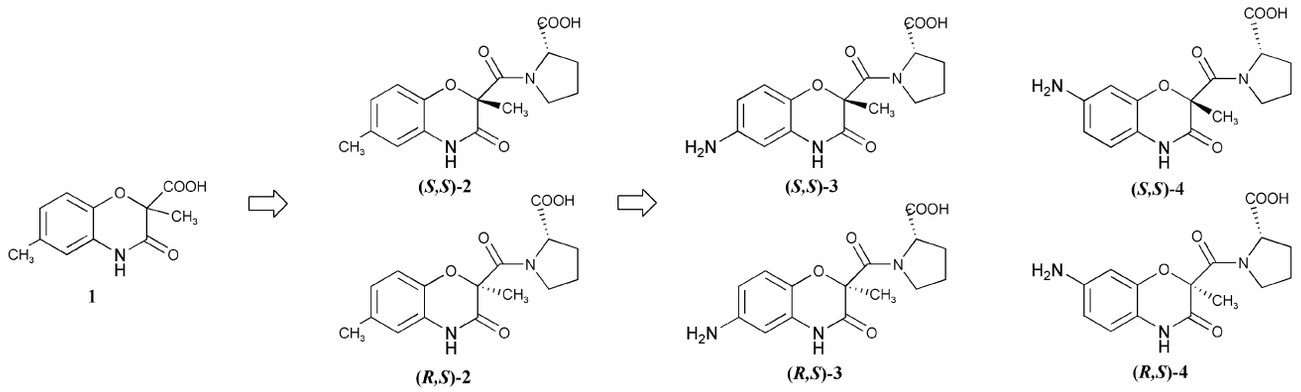

Fig. 1 The evolution of peptidomimetic templates (*S*,*S*)-**3**, (*R*,*S*)-**3**, (*S*,*S*)-**4** and (*R*,*S*)-**4**.

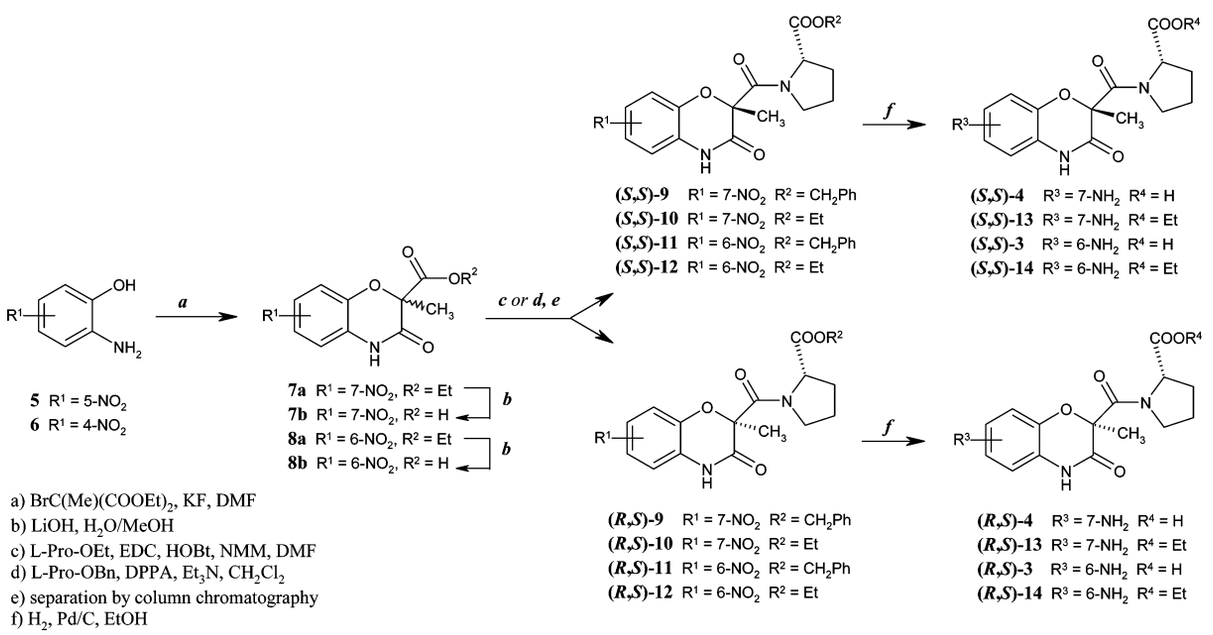

Fig. 2 Synthesis of peptidomimetic templates (*S,S*)-**3**, (*R,S*)-**3**, (*S,S*)-**4**, (*R,S*)-**4**, (*S,S*)-**13**, (*R,S*)-**13**, (*S,S*)-**14** and (*R,S*)-**14**.

building blocks for the construction of fibrinogen receptor antagonists. This would be achieved by coupling them with a benzamidine fragment which in target inhibitors would provide a basic moiety at a distance of 13 to 16 Å from the proline carboxylic group.

In this paper we report the synthesis and NMR conformational analysis of templates **3** and **4**, their elaboration to conformationally tailored fibrinogen receptor antagonists **15**–**18** and biological evaluation of target compounds supporting the rationale of the applied strategy.

Results and discussion

Chemistry

Ethyl 2-methyl-7-nitro-3-oxo-3,4-dihydro-2*H*-benzoxazine-2-carboxylate (**7a**) and ethyl 2-methyl-6-nitro-3-oxo-3,4-dihydro-2*H*-benzoxazine-2-carboxylate (**8a**) were synthesized from 2-aminophenols **5** and **6** according to procedures described previously.[21] Alkaline hydrolysis was then performed to produce the corresponding carboxylic acids **7b** and **8b**[21] which were subsequently coupled with (*S*)-proline benzyl ester, using DPPA as a coupling agent to give **9** and **11**, or with (*S*)-proline ethyl ester, using EDC and HOBT to give compounds **10** and **12** as diastereomeric mixtures. Column chromatography on silica gel was used to separate the *S,S* and *R,S* diastereomers of **9**–**12**. These were catalytically hydrogenated using palladium on activated charcoal to produce (2*S*)-1-[(2*R*/*S*)-(6/7-amino-2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benxazin-2-yl)carbonyl]-2-pyrrolidinecarboxylates (*S*,*S*)-**3**, (*R*,*S*)-**3**, (*S*,*S*)-**4**, (*R*,*S*)-**4** and ethyl (2*S*)-1-[(2*R*/*S*)-(6/7-amino-2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl)carbonyl]-2-pyrrolidinecarboxylates (*S*,*S*)-**13**, (*R*,*S*)-**13**, (*S*,*S*)-**14** and (*R*,*S*)-**14**, respectively (Fig. 2). The pure diastereomers of amines **13** and **14** were coupled with 4-cyanobenzoyl chloride, whereupon the cyano group of the resulting intermediates was transformed to an amidine functionality under the conditions of the Pinner reaction[22] to give the esters (*S*,*S*)-**15**, (*R*,*S*)-**15**, (*S*,*S*)-**17** and (*R*,*S*)-**17**. After alkaline hydrolysis, (*S*,*S*)-**16**, (*R*,*S*)-**16**, (*S*,*S*)-**18** and (*R*,*S*)-**18** were obtained as potential fibrinogen receptor antagonists (Fig. 3).

NMR studies. The conformational study of eight diastereomerically pure derivatives of *N*-[(2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl)carbonyl]-(*S*)-proline, *i.e.* benzyl esters of 6-nitro derivative [(*S*,*S*)-**11**, (*R*,*S*)-**11**], benzyl esters of 7-nitro derivative [(*S*,*S*)-**9**, (*R*,*S*)-**9**], and free carboxylates of 6-amino- and 7-amino derivatives [(*S*,*S*)-**3**, (*R*,*S*)-**3**, (*S*,*S*)-**4**, (*R*,*S*)-**4**], was performed by 2D NMR methods in dimethylsulfoxide. First, the proton NMR spectra were assigned using DFQ–COSY, HSQC and HMBC experiments in combination with ^{13}C broad band decoupled and DEPT spectra.

In the proton NMR spectra, the *S*,*S* diastereomers of **3**,**4**,**9** and **11** exhibited only one set of signals and, in the NOESY spectra, strong NOEs between δCH_2Pro/2-Me and δCH_2Pro/8-H, both evidencing the existence of only the *trans* conformation of the proline amide bond. For diastereomers of 6- and 7-amino compounds **3** and **4**, additional NOEs were observed

Table 1 The ratio of *trans* and *cis* isomers in (*R*,*S*)-**3**, (*R*,*S*)-**4**, (*R*,*S*)-**9**, (*R*,*S*)-**11**, (*R*,*S*)-**15**, (*R*,*S*)-**16**, (*R*,*S*)-**17** and (*R*,*S*)-**18**[a]

	(*R*,*S*)-**3**	(*R*,*S*)-**4**	(*R*,*S*)-**9**	(*R*,*S*)-**11**	(*R*,*S*)-**15**	(*R*,*S*)-**16**	(*R*,*S*)-**17**	(*R*,*S*)-**18**
Average ratio *trans*/*cis*	100 : 58	100 : 54	100 : 21	100 : 26	100 : 48	100 : 48	100 : 40	100 : 35
% *trans*	63	65	83	79	68	68	71	74
% *cis*	37	35	17	21	32	32	29	26

[a] Ratios were determined by integration of two sets of well resolved signals of *trans* and *cis* isomers in ^{1}H-NMR spectra in dimethylsulfoxide-d_6 solution.

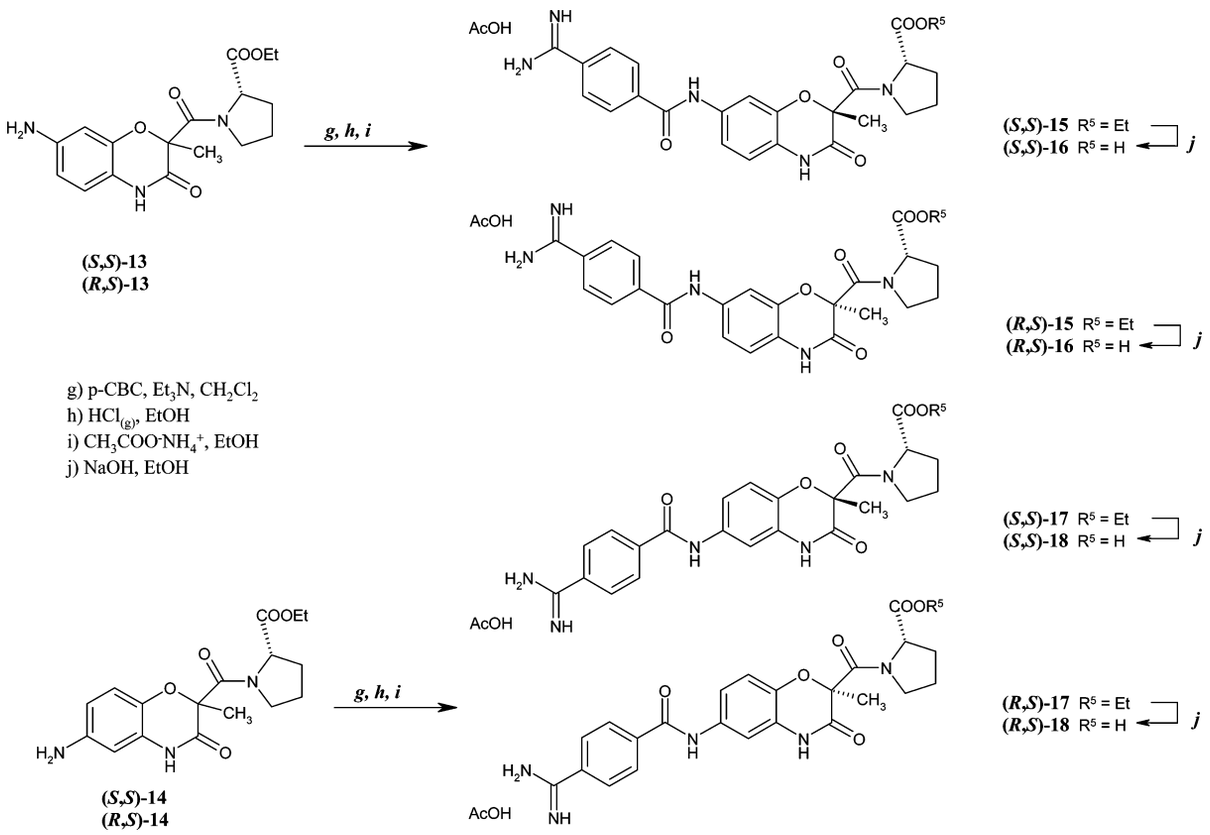

Fig. 3 The synthesis of diastereomers of potential fibrinogen receptor antagonists **15**,**16**,**17** and **18**.

between the pyrrolidine protons and the protons of the benzoxazinone ring, indicating that a limited rotation about the C2–C(O)Pro bond and/or puckering of the pyrrolidine ring could occur due to long range electrostatic interaction between the amino and carboxylate groups.

The 2*R*,2*S* diastereomers of **3**,**4**,**9** and **11** exhibited in their ^{1}H-NMR spectra two sets of signals. The major one exhibited in its NOE spectrum NOE connectivities between δCH_2Pro/2-Me and δCH_2Pro/8-H, attributed to the *trans*-conformer, whereas the minor set of signals, based on analogy with the previous results,[8] was assigned to the *cis*-conformer. The proportions of the *trans*- and *cis*-conformers, deduced as an average integral ratio of major and minor signals of 2-Me, NH, α–H, δ–H and OCH_2, were found to be 63–83% *trans* and 17–37% *cis* (Table 1). Interestingly, the ratio of the *cis* proline amide bond was higher in compounds possessing a free proline carboxylic group and an aromatic amino group, suggesting that a weak electrostatic interaction between the carboxylic and amino group might be responsible for a distortion of the ω dihedral angle leading to a higher proportion of the *cis* conformers.

Pyrrolidine ring puckering in *trans*-(*S*,*S*)-**3**, *trans*-(*S*,*S*)-**4**, *trans*-(*S*,*S*)-**9**, *cis*/*trans*-(*R*,*S*)-**9** and *trans*-(*S*,*S*)-**11** was studied by analysis of vicinal $^3J_{H,H}$ coupling constants, obtained by simulation of individual spectra. The resulting $^3J_{H,H}$ coupling constants (presented in Table 1 in the ESI †) (Fig. 4), have been interpreted in terms of a two-state North ⇔ South pseudorotational equilibrium, using the PSEUROT program.[23] In the

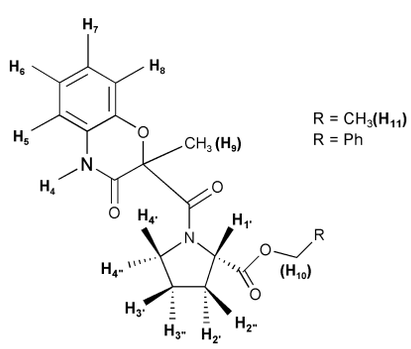

Fig. 4 Proton numbering used in conformational analysis and in assigning NMR spectra.

iterative optimization procedure the geometry (expressed by the phase angle of pseudorotation, *P*, and the maximum puckering amplitude, Ψ_m) of each of the conformers was freely optimized and the populations of North and South pseudorotamers were varied to obtain the best fit between experimental and calculated coupling constants. Agreement between experimental and back-calculated coupling constants was monitored by calculation of the root-mean-square deviation, which was found to be below 0.5 Hz. This conformational analysis led to the conclusion that the pyrrolidine rings are involved in a conformational equilibrium between a form close to the C2′-*exo* ($P \approx -20°$, $\Psi_m \approx 65°$) canonical form and a conformation between C4′-*endo* and C4′-*endo*-*N*δ-*exo* twist canonical forms

Table 2 IC_{50} values of diastereomers of compounds **15**–**18** and of RGDS as a reference compound for inhibition of fibrinogen binding to the fibrinogen receptor

Compound	R^5	Distance/nm	ω/degrees	IC_{50}/μM
RGDS				1.31 ± 0.06
(*S*,*S*)-**15**	Et			6.79 ± 1.58
(*S*,*S*)-**16**	H	1.580	177.3	0.51 ± 0.43
(*R*,*S*)-**15**	Et			4.75 ± 0.45
(*R*,*S*)-**16**	H	1.110 (*trans*)	179.5	4.10 ± 0.83
		1.104 (*cis*)	5.0	
(*S*,*S*)-**17**	Et			6.48 ± 3.10
(*S*,*S*)-**18**	H	1.694	−174.8	6.89 ± 1.43
(*R*,*S*)-**17**	Et			13.83 ± 2.98
(*R*,*S*)-**18**	H	1.208 (*trans*)	174.2	104.22 ± 42.6
		1.475 (*cis*)	−16.4	

($P \approx 245°$, $\Psi_m \approx 51°$). The population of the above two conformers of the pyrrolidine rings was found to be approximately equal at 29° C in DMSO-d_6.

Biological activity. The biological activity of pure diastereomers of the target compounds **15**–**18** was evaluated in the integrin receptor assay.[24] The extent of competitive binding to the fibrinogen and vitronectin receptors in the presence of chemically derivatized natural agonist (biotinylated fibrinogen) was determined from the rate of chemiluminescence produced by horse-radish peroxidase attached to the biotin directed antibody. Compounds **15**–**18** inhibiting the binding of fibrinogen in the low micromolar range were selective and moderately potent fibrinogen receptor antagonists, when compared to therapeutically used tirofiban and eptifibatide. The IC_{50} values of diastereomers of compounds **15**–**18**, representing the concentration of an antagonist causing a 50% inhibition of binding of natural agonist fibrinogen, are listed in Table 2. In the preliminary screening, the estimated IC_{50} values for the binding of **15**–**18** to the vitronectin receptor were higher than 100 μM and are not presented here.

General discussion. Peptidomimetic compounds *trans*-(*S*,*S*)-**15**, *trans*-(*S*,*S*)-**16**, *trans*-(*S*,*S*)-**17** and *trans*-(*S*,*S*)-**18** were designed as conformationally tailored fibrinogen receptor antagonists, their well defined conformation being due to a defined absolute configuration at C2 and the due existence of a solely *trans* proline amide bond. Molecular modelling of compounds *trans*-(*S*,*S*)-**15**, *trans*-(*S*,*S*)-**16**, *trans*-(*S*,*S*)-**17** and *trans*-(*S*,*S*)-**18** revealed conformationally highly restricted structures with a proline ring folded almost perpendicularly over the benzoxazinone moiety and the COOR group located at the external side of a twisted structure (Fig. 1 in ESI). The distances between C-atoms of the *C*(NH)NH_2 and the proline *C*OOR (R = H, Et) groups were found to be 1.580 nm for *trans*-(*S*,*S*)-**15** or *trans*-(*S*,*S*)-**16** and 1.694 nm for *trans*-(*S*,*S*)-**17** or *trans*-(*S*,*S*)-**18**. The (*R*,*S*)-diastereomers of compounds **15**–**18** exist as mixtures of (mainly) *trans*- and *cis* isomers. As a consequence of the different absolute configuration at C2, in *trans*-(*R*,*S*) **15**–**18** the distance between the *C*(NH)NH_2 and the proline *C*OOR (R = H, Et) groups is shorter than in the corresponding *trans*-(*S*,*S*) isomers (1.110 nm in *trans*-(*R*,*S*)-**16** *vs*. 1.580 nm in *trans*-(*S*,*S*)-**16**, 1.208 nm in *trans*-(*R*,*S*)-**18** *vs*. 1.694 nm in *trans*-(*S*,*S*)-**18**). A pharmacophore model for antagonistic activity at the fibrinogen receptor, demands for an antagonist a slightly cup-shaped conformation of the scaffold with a distance of 1.3–1.8 nm between the anionic and cationic moieties.[9–11] Thus, the higher potency of *trans*-(*S*,*S*)-**16** *vs*. *cis*/*trans*-(*R*,*S*)-**16** and *trans*-(*S*,*S*)-**18** *vs*. *cis*/*trans*-(*R*,*S*)-**18** can be explained on the basis of different distances between the *C*(NH)NH_2 and *C*OOH groups. The relationship between potency and distance of the *C*(NH)NH_2 and *C*OOEt groups in isomers of esters **15** and **17** is, however, not straightforward, suggesting that other interactions, such as hydrogen bonding and hydrophobic interactions, could contribute to the binding of diastereomers of **15** and **17** to the fibrinogen receptor.

In conclusion, we have demonstrated that *N*-[(3,4-dihydro-2-methyl-3-oxo-2*H*-1,4-benzoxazin-2-yl)-2-carbonyl]-(*S*)-proline templates, in which the *cis*/*trans* ratio of the proline peptide bond can be tailored by the chirality of the acyl residue, can be used as conformationally defined scaffolds for constructing fibrinogen receptor antagonists. The conformationally defined *trans*-(*S*,*S*)-**16** and *trans*-(*S*,*S*)-**18**, which possess a moderate antagonistic potency at the fibrinogen receptor, verify the concept of chirality-induced tailoring of the proline peptide bond conformation as a promising tool in the design of peptidomimetics.

Experimental

Chemicals from Aldrich Chemical Co., Fluka, Merck, Kemika and Jannsen were used without further purification. Anhydrous dichloromethane, *N*,*N*-dimethylformamide and triethylamine were prepared according to standard procedures.[25] Analytical TLC was performed on Merck silica gel 60 F_{254} plates (0.25 mm) and components were visualized under ultraviolet light. Column chromatography was carried out on silica gel 60 (particle size 240–400 mesh). Melting points were determined on a Reichert hot stage microscope and are uncorrected. ^{1}H-NMR spectra were recorded on a Bruker AVANCE DPX_{300} spectrometer in $CDCl_3$ or DMSO-d_6 solution, with TMS as the internal standard. NMR spectra (DFQ-COSY, HSQC, HMBC, ^{13}C broad band decoupled and DEPT spectra) for conformational analysis were performed on a Varian Unity Inova 600 and a Varian Unity Inova 300, using DMSO-d_6 solution with TMS as the internal standard at 302 K and 298 K. *J* values are given in Hz. Conformational analysis of the pyrrolidine moiety was performed by the computer program PSEUROT[26] with the use of λ electronegativities for the substituents along H–C–C–H fragments and the six parameter set for the generalized Karplus–type equation.[27] The following λ electronegativity values were used: 0.0 for H, 0.6 for C1′, 0.7 for C3′, 1.19 for N, 0.42 for COO and 0.75 for C4′. The analysis of $^3J_{HH}$ coupling constants consists of three standard translation steps. The first step translates experimental proton–proton coupling constants to proton–proton torsion angles and is covered by the generalized Karplus–Altona equation. The second step is the translation of proton–proton torsion angles into the corresponding endocyclic torsion angles, and is formulated with the set of linear equations $\Phi_{HH} = A\nu_j + B$. Φ_{HH} is the torsion angle between two vicinal protons and ν_j is the corresponding endocyclic torsion angle. The third step of translating endocyclic torsion angles into the pseudorotational parameters is described by a simple cosine function $\nu_j = \Psi_m * \cos[P + (j–2) * 4\pi/5]$, where *P* is the phase angle of pseudorotation and Ψ_m is the maximum puckering amplitude. In the following optimization procedure the geometries and populations of North and South pseudorotamers were varied to obtain the best fit between experimental and calculated coupling constants. IR spectra were obtained using a Perkin-Elmer 1600 FT-IR spectrometer. Microanalyses were performed on a Perkin-Elmer C, H, N analyzer 240 C. HPLC analyses were performed on Agilent Technologies HP 1100 instrument with G1365B UV-VIS detector using a Eurospher C_{18} column (4.6 × 250 mm). Eluent consisted of 0.1% trifluoroacetic acid in water (40%) and methanol (60%). Mass spectra were obtained using a VG-Analytical Autospec Q mass spectrometer. All reported yields are yields of purified products.

General procedure for preparing ethyl 2-methyl-(6/7)-nitro-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-2-carboxylates 7a and 8a

A suspension of potassium fluoride (2.81 g, 48.4 mmol) and diethyl 2-bromo-2-methyl-malonate (4.92 g, 19.4 mmol) in

N,*N*-dimethylformamide (40 mL) was stirred for 15 min at room temperature. 2-Amino-4/5-nitrophenol (3.00 g, 19.4 mmol) was then added and the reaction mixture heated at 60 °C for 6 h. When the cooled reaction mixture was poured into ice-cold water (50 g), a precipitate formed, which was filtered off, washed with water and recrystallized from ethanol (90 mL).

Ethyl 2-methyl-7-nitro-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-2-carboxylate 7a

The procedure described yielded **7a** (4.05 g, 75%) as violet crystals, mp 190–195 °C (lit.,[21] 194–195 °C); ν_{max}(KBr)/cm^{-1} 3087, 2945, 1748, 1695, 1605, 1540, 1340, 1237, 1123, 1011, 825, 743 and 550; δ_H(300 MHz; $CDCl_3$; Me_4Si) 1.21 (3H, t, *J* 7.2, CH_2CH_3), 1.93 (3H, s, CH_3), 4.13–4.29 (2H, m, CH_2CH_3), 6.93 (1H, d, $J_{5,6}$ 8.7, H_5), 7.95 (1H, dd, $J_{6,8}$ 2.3, $J_{5,6}$ 8.7, H_6) and 7.99 (1H, d, $J_{6,8}$ 2.3, H_8); *m*/*z* (EI): 280 (M^+, 37%) and 207 (100).

General procedure for preparing 2-methyl-(6/7)-nitro-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-2-carboxylic acids 7b and 8b

Lithium hydroxide (0.389 g, 16.3 mmol) was added to a solution of ethyl 2-methyl-(6/7)-nitro-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-2-carboxylate **7a** or **7b** (3.5 g, 12.5 mmol) in a mixture of methanol and water (50 mL, 50% v/v) and the mixture was stirred for 12 h. The methanol was evaporated under reduced pressure and the aqueous phase washed with diethyl ether (3 × 30 mL), acidified with 1 M HCl to pH 2–3 and extracted with ethyl acetate (5 × 30 mL). The combined organic fractions were dried with sodium sulfate and the solvent evaporated under reduced pressure to produce a crystalline product.

2-Methyl-7-nitro-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-2-carboxylic acid 7b

The procedure described yielded **7b** (3.06 g, 97%) as violet crystals, mp 180–184 °C (lit.,[21] 183–185 °C); ν_{max} (KBr)/cm^{-1} 3494, 3098, 1698, 1606, 1538, 1342, 1250, 1136, 832 and 745; δ_H(300 MHz; $CDCl_3$; Me_4Si) 1.94 (3H, s, CH_3), 6.91 (1H, d, $J_{5,6}$ 8.7, H_5), 7.92 (1H, dd, $J_{6,8}$ 2.3, $J_{5,6}$ 8.7, H_6) and 7.99 (1H, d, $J_{6,8}$ 2.3, H_8); *m*/*z* (EI): 252 (M^+, 53%) and 208 (100).

General procedure for preparing benzyl (2*S*)-1-{[(2*R*/*S*)-2-methyl-(6/7)-nitro-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl]carbonyl}-2-pyrrolidinecarboxylates (*S*,*S*)-9, (*R*,*S*)-9, (*S*,*S*)-11 and (*R*,*S*)-11

2-Methyl-(6/7)-nitro-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-2-carboxylic acid **7b** or **8b** (0.86 g, 3.40 mmol) and benzyl (2*S*)-2-pyrrolidinecarboxylate hydrochloride (0.83 g, 3.40 mmol) were dissolved in anhydrous *N*,*N*-dimethylformamide (20 mL) and DPPA (0.92 mL, 4.30 mmol) and triethylamine (1.2 mL, 8.60 mmol) were added at 0 °C. The reaction mixture was stirred first for 1 h at 0 °C and then for 60 h at room temperature. Ethyl acetate (50 mL) was then added and the reaction mixture washed with 10% citric acid (5 × 20 mL), water (3 × 20 mL), a saturated solution of sodium hydrogen carbonate (3 × 20 mL), water (3 × 20 mL) and a saturated solution of sodium chloride (2 × 20 mL). The organic solution was dried over sodium sulfate and the solvent evaporated under reduced pressure to give a crystalline product. Separation of the diastereomers was achieved by column chromatography on silica gel with diethyl ether as eluant.

Benzyl (2*S*)-1-{[(2*S*)-2-methyl-7-nitro-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl]carbonyl}-2-pyrrolidinecarboxylate (*S*,*S*)-9

The procedure described yielded (*S,S*)-**9** (0.30 g, 20%) as yellow crystals, mp 89–95 °C; $[\alpha]_D^{20}$ −43.8 (*c* 0.513 in MeOH); Found: C, 60.25; H, 4.7; N, 9.5. $C_{22}H_{21}N_3O_7$ requires C, 60.1; H, 4.8, N, 9.6%; ν_{max}(KBr)/cm^{-1} 3240, 1726, 1640, 1531, 1448, 1325, 1156, 1041, 754 and 697; δ_H(300 MHz; $CDCl_3$; Me_4Si) 1.66 (3H, s, CH_3), 1.73–1.83 (1H, m, $J_{1',2''}$ 8.8, $J_{2',2''}$ 12.7, $J_{2'',3'}$ 7.5, $J_{2'',3''}$ 7.4, $H_{2''}$), 1.85–1.96 (2H, m, $J_{2',3'}$ 6.7, $J_{2',3''}$ 6.3, $J_{3',3''}$ 12.2, $J_{3',4'}$ 7.0, $J_{3',4''}$ 6.9, $J_{3'',4'}$ 6.4, $J_{3'',4''}$ 6.4, $H_{3'}$, $H_{3''}$), 2.06–2.13 (1H, m, $J_{1',2'}$ 5.0, $H_{2'}$), 3.74–3.79 (2H, m, $J_{4',4''}$ 10.7, $H_{4'}$, $H_{4''}$), 4.29 (1H, dd, $J_{1',2'}$ 5.0, $J_{1',2''}$ 8.8, $H_{1'}$), 4.67 and 4.96 (each 1H, d, *J* 12.6, CH_2Ph), 7.10 (1H, d, $J_{5,6}$ 8.7, H_5), 7.33–7.39 (5H, m, Ph), 7.92 (1H, d, $J_{6,8}$ 2.4, H_8) and 7.96 (1H, dd, $J_{6,8}$ 2.4, $J_{5,6}$ 8.7, H_6); *m*/*z* (EI): 439 (M^+, 63%) and 91 (100).

General procedure for preparing ethyl (2*S*)-1-{[(2*R*/*S*)-2-methyl-(6/7)-nitro-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl]carbonyl}-2-pyrrolidine-carboxylates (*S*,*S*)-10, (*R*,*S*)-10, (*S*,*S*)-12 and (*R*,*S*)-12

2-Methyl-(6/7)-nitro-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-2-carboxylic acid **7b** or **8b** (2.52 g, 10.0 mmol) and ethyl (2*S*)-2-pyrrolidinecarboxylate hydrochloride (1.80 g, 10.0 mmol) were dissolved in anhydrous *N*,*N*-dimethylformamide (20 mL). 1-Hydroxybenzotriazole (1.62 g, 12.0 mmol) was added and the pH adjusted to 8 with *N*-methylmorpholine before adding EDC (2.59 g, 13.5 mmol). The reaction mixture was stirred overnight at room temperature. The solvent was evaporated under reduced pressure and the oily residue dissolved in dichloromethane (150 mL), washed with 10% citric acid (2 × 40 mL) and saturated sodium hydrogen carbonate solution (2 × 40 mL). The organic solution was dried over sodium sulfate and the solvent evaporated under reduced pressure to yield an oily product. The separation of the diastereomers was achieved by column chromatography on silica gel with diethyl ether as an eluant.

Ethyl (2*S*)-1-{[(2*S*)-2-methyl-7-nitro-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl]carbonyl}-2-pyrrolidinecarboxylate (*S*,*S*)-10

The procedure described yielded (*S,S*)-**10** (0.302 g, 8%) as pale yellow crystals, mp 137–139 °C; $[\alpha]_D^{20}$ −37.9 (*c* 0.100 in MeOH); Found: C, 53.0; H, 5.5; N, 10.55. $C_{17}H_{19}N_3O_7 \times 1/2H_2O$ requires: C, 52.85; H, 5.2; N, 10.9%; ν_{max} (KBr)/cm^{-1} 3088, 2944, 1750, 1696, 1605, 1537, 1340, 1237, 1124, 878, 826 and 744; δ_H(300 MHz; DMSO-d_6; Me_4Si) 1.16 (3H, t, *J* 7.0, CH_2CH_3), 1.68–1.78 (1H, m, $H_{2''}$), 1.71 (3H, s, CH_3), 1.81–1.99 (2H, m, $H_{3'}$, $H_{3''}$), 2.03–2.15 (1H, m, $H_{2'}$), 3.74–3.82 (2H, m, $H_{4'}$, $H_{4''}$), 4.01–4.12 (2H, m, CH_2CH_3), 4.19 (1H, dd, J_1 8.7, J_2 4.9, $H_{1'}$), 7.11 (1H, d, $J_{5,6}$ 9.4, H_5), 7.96–8.00 (2H, m, H_6, H_8) and 11.54 (1H, s, N*H*); *m*/*z* (FAB): 378 (MH^+,100%), 362 (10), 304 (30), 207 (22), 142 (45) and 70 (51).

General procedure for preparing (2*S*)-1-{[(2*R*/*S*)-(6/7)-amino-2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl]carbonyl}-2-pyrrolidinecarboxylic acids (*S*,*S*)-3, (*R*,*S*)-3, (*S*,*S*)-4 and (*R*,*S*)-4

The solution of benzyl (2*S*)-1-{[(2*R*/*S*)-2-methyl-(6/7)-nitro-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl]-carbonyl}-2-pyrrolidinecarboxylate (*S*,*S*)-**9**, (*R*,*S*)-**9**, (*S*,*S*)-**11** or (*R*,*S*)-**11** (0.15 g, 0.341 mmol) in anhydrous methanol was catalytically hydrogenated in the presence of palladium on activated charcoal (10% by weight) by hydrogen bubbling into the reaction mixture for 2 hours followed by stirring the reaction mixture under a hydrogen atmosphere for 12 hours. The catalyst was filtered off and the solvent evaporated under reduced pressure to give a crystalline product.

(2*S*)-1-{[(2*S*)-7-Amino-2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl]carbonyl}-2-pyrrolidinecarboxylic acid (*S*,*S*)-4

The procedure described yielded (*S,S*)-**4** (0.079 g, 73%) as pink crystals, mp 171–173 °C; $[\alpha]_D^{20}$ −52.3 (*c* 0.435 in MeOH); ν_{max}(KBr)/cm^{-1} 3446, 1684, 1652, 1559, 1519, 1456, 1260 and 1176; δ_H(300 MHz; DMSO-d_6; Me_4Si) 1.54 (3H, s, CH_3), 1.71–1.78 (1H, m, $J_{1',2''}$ 8.7, $J_{2',2''}$ −12.7, $J_{2'',3'}$ 7.6, $J_{2'',3''}$ 7.0, $H_{2''}$),

1.79–1.98 (2H, m, $J_{2',3'}$ 6.7, $J_{2',3''}$ 6.2, $J_{3',3''}$ −12.4, $J_{3',4'}$ 6.6, $J_{3',4''}$ 6.3, $J_{3'',4'}$ 6.8, $J_{3'',4''}$ 7.0, $H_{3'}$, $H_{3''}$), 2.01–2.10 (1H, m, $J_{1',2'}$ 4.8, $H_{2'}$), 2.62 (2H, s, NH_2), 3.61–3.74 (2H, m, $J_{4',4''}$ −10.4, $H_{4'}$, $H_{4''}$), 4.14 (1H, dd, $J_{1',2'}$ 4.8, $J_{1',2''}$ 8.7, $H_{1'}$), 6.21 (1H, dd, $J_{6,8}$ 2.2, $J_{5,6}$ 8.3, H_6), 6.26 (1H, d, $J_{6,8}$ 2.2, H_8), 6.58 (1H, d, $J_{5,6}$ 8.3, H_5) and 10.40 (1H, s, N*H*); *m*/*z* (EI): 319 (M^+, 35%) and 178 (100). HR-MS Found: 319.117300, $C_{15}H_{17}N_3O_5$ requires: 319.116821.

General procedure for preparing ethyl (2*S*)-1-{[(2*R*/*S*)-(6/7)-amino-2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl]-carbonyl}-2-pyrrolidine-carboxylates (*S*,*S*)-13, (*R*,*S*)-13, (*S*,*S*)-14 and (*R*,*S*)-14

The solution of ethyl (2*S*)-1-{[(2*R*/*S*)-2-methyl-(6/7)-nitro-3-oxo-3,4-dihydro-*2H*-1,4-benzoxazin-2-yl]carbonyl}-2-pyrrolidinecarboxylate (*S*,*S*)-**10**, (*R*,*S*)-**10**, (*S*,*S*)-**12** or (*R*,*S*)-**12** (0.377 g, 1.00 mmol) in anhydrous ethanol (40 mL) was catalytically hydrogenated in the presence of palladium on activated charcoal (10% by weight) by stirring the reaction mixture under hydrogen atmosphere overnight. The catalyst was filtered off and the solvent evaporated under reduced pressure to give a crystalline product.

Ethyl (2*S*)-1-{[(2*S*)-7-amino-2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl]carbonyl}-2-pyrrolidinecarboxylate (*S*,*S*)-13

The procedure described yielded (*S*,*S*)-**13** (0.337 g, 97%) as white crystals, mp 114–117 °C; $[\alpha]_D^{20}$ −55.8 (*c* 0.100 in MeOH); Found: C, 58.5; H, 6.3; N, 11.8. $C_{17}H_{21}N_3O_5$ reqiures: C, 58.8; H, 6.1; N, 12.1%; ν_{max}(KBr)/cm^{-1} 3362, 2982, 1732, 1687, 1635, 1520, 1375, 1180, 1023, 808 and 619; δ_H(300 MHz; DMSO-d_6; Me_4Si) 1.18 (t, 3H, *J* 7.2, CH_2CH_3), 1.54 (s, 3H, CH_3), 1.72–1.94 (m, 3H, $H_{2''}$, $H_{3'}$, $H_{3''}$), 2.05–2.14 (m, 1H, $H_{2'}$), 3.64–3.77 (m, 2H, $H_{4'}$, $H_{4''}$), 4.04–4.11 (m, 2H, CH_2CH_3), 4.21 (dd, 1H, $J_{1',2'}$ 5.1, $J_{1',2''}$ 8.5, $H_{1'}$), 4.93 (s, 2H, NH_2), 6.21 (dd, 1H, $J_{6,8}$ 2.3, $J_{5,6}$ 8.3, H_6), 6.28 (d, 1H, $J_{6,8}$ 2.3, H_8), 6.58 (d, 1H, $J_{5,6}$ 8.3, H_5) and 10.42 (s, 1H, N*H*); *m*/*z* (FAB): 348 (MH^+, 100%), 274 (8), 205 (12), 177 (57), 154 (28), 142 (38) and 136 (23).

General procedure for preparing ethyl (2*S*)-1-{[(2*R*/*S*)-6/7-({4-[amino(imino)methyl]benzoyl}-amino)-2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl]carbonyl}-2-pyrrolidinecarboxylate acetates (*S*,*S*)-15, (*R*,*S*)-15, (*S*,*S*)-17 and (*R*,*S*)-17

To a stirred solution of the appropriate ethyl (2*S*)-1-[(2*R*/*S*)-((6/7)-amino-2-methyl-3-oxo-3,4-dihydro-*2H*-1,4-benzoxazin-2-yl)carbonyl]-2-pyrrolidinecarboxylate (*S*,*S*)-**13**, (*R*,*S*)-**13**, (*S*,*S*)-**14** or (*R*,*S*)-**14** (0.695 g, 2.00 mmol) and triethylamine (0.3 mL, 2.20 mmol) in anhydrous dichloromethane (30 mL) was added 4-cyanobenzoyl chloride (0.397 g, 2.4 mmol) in small portions at 0 °C. After the addition was complete, stirring was continued for four hours at room temperature. The reaction mixture was washed with 10% citric acid (2 × 50 mL) and saturated $NaHCO_3$ solution (2 × 50 mL), dried with sodium sulfate, filtered and the solvent evaporated under reduced pressure to give the corresponding ethyl (2*S*)-1-({(2*R*/*S*)-(6/7)-[(4-cyanobenzoyl)amino]-2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl}carbonyl)-2-pyrrolidinecarboxylate.

To a solution of the respective ethyl (2*S*)-1-({(2*R*/*S*)-6/7-[(4-cyanobenzoyl)amino]-2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl}carbonyl)-2-pyrrolidine-carboxylate (0.476 g, 1.00 mmol) in anhydrous ethanol (20 mL) was introduced gaseous hydrogen chloride at 0 °C for 30 minutes. The reaction mixture was stirred overnight at room temperature. The solvent was evaporated under reduced pressure and washed 3–4 times with diethyl ether to give a crystalline product. This was dissolved in anhydrous ethanol (15 mL), ammonium acetate (0.23 g, 3.00 mmol) was added, and the reaction mixture was stirred for 2–3 days at room temperature. The solvent was evaporated under reduced pressure and the product purified by preparative thin layer chromatography using *n*-BuOH/H_2O/AcOH (5 : 4 : 1) as eluent.

Ethyl (2*S*)-1-({(2*S*)-7-[(4-cyanobenzoyl)amino]-2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl}-carbonyl)-2-pyrrolidine-carboxylate

The procedure described yielded 0.943 g (99%) of yellow crystals, mp 120–124 °C; $[\alpha]_D^{20}$ −88.5 (*c* 0.100 in MeOH); Found: C, 63.2; H, 5.2; N, 11.5, $C_{25}H_{24}N_4O_6$ requires: C, 63.0; H, 5.1; N, 11.7%; ν_{max}(KBr)/cm^{-1} 3280, 2981, 2231, 1700, 1517, 1374, 1178, 1019, 856 and 760; δ_H(300 MHz; DMSO-d_6; Me_4Si) 1.18 (t, 3H, *J* 7.2, CH_2CH_3), 1.63 (s, 3H, CH_3), 1.72–1.79 (m, 1H, $H_{2''}$), 1.83–1.98 (m, 2H, $H_{3'}$, $H_{3''}$), 2.07–2.14 (m, 1H, $H_{2'}$), 3.71–3.82 (m, 2H, $H_{4'}$, $H_{4''}$), 4.02–4.12 (m, 2H, CH_2CH_3), 4.21 (dd, 1H, $J_{1',2'}$ 4.7, $J_{1',2''}$ 8.9, $H_{1'}$), 6.90 (d, 1H, $J_{5,6}$ 8.5, H_5), 7.39 (dd, 1H, $J_{5,6}$ 8.5, $J_{6,8}$ 2.3, H_6), 7.59 (d, 1H, $J_{6,8}$ 2.3, H_8), 8.02 (d, 2H, *J* 8.7, 4-CN-Ar-H), 8.09 (d, 2H, *J* 8.7, 4-CN-Ar-H), 10.44 (s, 1H, N*H*) and 10.89 (s, 1H, 4-CN-ArCON*H*-); *m*/*z* (FAB): 477 (MH^+, 100%), 403 (14), 306 (50), 154 (53), 142 (63), 71 (61) and 57 (87).

Ethyl (2*S*)-1-{[(2*S*)-7-({4-[amino(imino)methyl]benzoyl}amino)-2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl]carbonyl}-2-pyrrolidinecarboxylate acetate (*S*,*S*)-15

The procedure described yielded (*S*,*S*)-**15** (0.238 g, 43%) as yellow crystals, mp 195–200 °C; $[\alpha]_D^{20}$ −91.6 (*c* 0.100 in MeOH); Found: C, 56.5; H, 5.9; N, 12.1, $C_{27}H_{31}N_5O_8 \times H_2O$ requires: C, 56.7; H, 5.8; N, 12.3%; ν_{max}(KBr)/cm^{-1} 3416, 1646, 1558, 1519, 1416, 1180, 1019 and 654; δ_H(300 MHz; DMSO-d_6; Me_4Si) 1.18 (t, 3H, *J* 7.2, CH_2CH_3), 1.63 (s, 3H, CH_3), 1.79 (s, 3H, CH_3-COOH), 1.70–1.98 (m, 3H, $H_{2''}$, $H_{3'}$, $H_{3''}$), 2.05–2.15 (m, 1H, $H_{2'}$), 3.69–3.82 (m, 2H, $H_{4'}$, $H_{4''}$), 4.03–4.15 (m, 2H, CH_2CH_3), 4.21 (dd, 1H, $J_{1',2'}$ 4.9, $J_{1',2''}$ 8.7, $H_{1'}$), 6.92 (d, 1H, $J_{5,6}$ 8.4, H_5), 7.43 (dd, 1H, $J_{5,6}$ 8.4, $J_{6,8}$ 2.2, H_6), 7.63 (d, 1H, $J_{6,8}$ 2.2, H_8), 7.93 (d, 2H, *J* 8.3, 4–CN–Ar–H), 8.11 (d, 2H, *J* 8.3, 4–CN–Ar–H), and 10.52 (s, 1H, N*H*); [C(NH)NH_3^+ gives together with H_2O a broad signal at 3.0–4.2 ppm; a signal of 4-CN-ArCON*H*- was not detected]; *m*/*z* (FAB): 494 (MH^+, 50%), 413 (7), 329 (19), 176 (84), 154 (76), 136 (68), 71 (81) and 55 (100).

General procedure for preparing (2*S*)-1-{[(2*R*/*S*)-(6/7)-({4-[amino(imino)methyl]benzoyl}amino)-2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl]-carbonyl}-2-pyrrolidinecarboxylic acid acetates (*S*,*S*)-16, (*R*,*S*)-16, (*S*,*S*)-18 and (*R*,*S*)-18

Ethyl (2*S*)-1-{[(2*R*/*S*)-(6/7)-({4-[amino(imino)methyl]benzoyl}-amino)-2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl]-carbonyl}-2-pyrrolidinecarboxylate acetate (*S*,*S*)-**15**, (*R*,*S*)-**15**, (*S*,*S*)-**17** or (*R*,*S*)-**17** (0.278 g, 0.5 mmol) was dissolved in ethanol (10 mL), 2 M NaOH solution (0.5 mL) was added and the reaction mixture stirred at room temperature overnight. The solvent was evaporated under reduced pressure to half the volume, AcOH (2–3 equivalents) was added and the solution cooled at 4 °C to form a crystalline precipitate.

(2*S*)-1-{[(2*S*)-7-({4-[amino(imino)methyl]benzoyl}-amino)-2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl]carbonyl}-2-pyrrolidinecarboxylic acid acetate (*S*,*S*)-16

The procedure described yielded (*S*,*S*)-**16** (0.137 g, 52%) as pale yellow crystals, mp > 300 °C; $[\alpha]_{546}^{20}$ −6.4 (*c* 0.100 in MeOH); ν_{max}(KBr)/cm^{-1} 3446, 1638, 1560, 1412, 1282, 1021, 814 and 644; δ_H(300 MHz; DMSO-d_6; Me_4Si) 1.49 (s, 3H, CH_3), 1.86 (s, 3H, CH_3COOH), 1.57–1.94 (m, 3H, $H_{3'}$, $H_{3''}$, $H_{2''}$), 4.18 (br s, 1H, $H_{1'}$), 6.82 (d, 1H, $J_{5,6}$ 8.6, H_5), 7.10–7.13 (m, 1H, H_6), 7.54 (br s, 1H, H_8), 7.68–7.77 (m, 4H, 4–CN–Ar–H) and 10.46 (br s, 1H, N*H*); [C(NH)NH_3^+ gives together with H_2O a broad signal at 3.0–4.2 ppm]; signals of 4-CN-ArCON*H*- and COO*H* were not detected; signals for $H_{2'}$, $H_{4'}$, $H_{4''}$ are under the corresponding signal for water]; *m*/*z* (FAB): 466 (MH^+, 1%), 391 (7),

307 (22), 289 (12), 154 (100), 136 (76), 107 (28), 71 (46) and 55 (50); HPLC: peak area > 95%.

For data of other compounds see ESI †.

The receptor binding assay

Biotinylation of fibrinogen. 5 mL of 0.3 M NaCl solution was added to human fibrinogen (100 mg) and incubated at 30 °C until complete dissolution. 300 μL of the fibrinogen solution and 600 μL of 1 M $NaHCO_3$ were diluted with bidistilled water to obtain a solution with a final fibrinogen concentration of 1 mg mL^{-1}. (+)-Biotin *N*-succinimidyl ester solution (2 mL) in *N*,*N*-dimethylformamide (1 mg per 1 mL) was added and incubated for 1.5 h at 30 °C. After dialysis of the fibrinogen solution against Tris buffer (20 mM Tris, 150 mM NaCl, pH = 7.4), the solution was centrifuged for 5 min at 5000 rpm. The supernatant was stabilised with Tween 20 (0.005%) and stored at 4 °C.

Solid-phase receptor binding assay. Integrin (fibrinogen receptor (1 μg mL^{-1}) or vitronectin receptor (0.2 μg mL^{-1})) in Tris buffer solution (20 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, pH = 4) was applied on a Lumitrac 600 96-well microtiter plate (100 μL per well) and incubated at 4 °C overnight. The integrin solution was poured away and the free binding sites blocked with 1% BSA Tris buffer solution (200 μL per well) for 1 hour at room temperature, followed by two-times washing with Tris buffer containing 0.01% Tween 20. 100 μL of different dilutions of the potential integrin antagonists (200 μM, 60 μM, 20 μM, 6 μM, 2 μM, 0.6 μM, 0.2 μM, 0.06 μM and 0.02 μM in Tris) were applied, followed by 100 μL biotinylated fibrinogen solution and 2h incubation at room temperature. The plates were washed twice with Tris buffer containing 0.01% Tween 20. 100 μL biotin directed horseradish peroxidase conjugated goat antibodies (Calbiochem; 1 : 1000 dilution in Tris buffer containing 0.1% BSA) were added to each well and the plate incubated for 1 hour at room temperature. After washing the plate three times with Tris buffer containing 0.01% Tween 20, the chemiluminescence reagent (50 μL per well) was added and within 15 minutes the light emission was measured with Tecan GENios using Magellan (Version 3.0) software. The chemiluminescence intensity data was fitted to a dose-response curve, from which the concentration of the antagonists causing 50% inhibition of fibrinogen binding (the IC_{50} value) was obtained. The data were processed by Excel and Microcal Origin.

Molecular modelling. The structures of *trans*-(*S*,*S*)-**16**, *trans*-(*R*,*S*)-**16**, *cis*-(*R*,*S*)-**16**, *trans*-(*S*,*S*)-**18**, *trans*-(*R*,*S*)-**18** and *cis*-(*R*,*S*)-**18** were drawn in ISIS Draw 2.3. The structures were transferred to HyperChem™ (Release 5.01 for Windows, Hypercube, Inc. 1996) and the 'add hydrogens and model build' function was carried out, followed by geometry optimisation using the Molecular mechanics force field method and Polak-Ribiere (Conjugate gradient) minimisation algorithm (RMS gradient of 5 exp(-5) kcal/(Amol)). The obtained minimized/low-energy conformations were compared to the data obtained from NOESY experiments. Additionally, the stereochemistry of benzoxazinone C-2 and proline C-δ and the geometry of proline amide bond were checked. The distances between the anionic and cationic moieties in *trans*-(*S*,*S*)-**16**, *trans*-(*R*,*S*)-**16**, *cis*-(*R*,*S*)-**16**, *trans*-(*S*,*S*)-**18**, *trans*-(*R*,*S*)-**18** and *cis*-(*R*,*S*)-**18** were measured and compared to the known pharmacophore.

Abbreviations

EDC: 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HOBT: 1-hydroxybenzotriazole hydrate; DPPA: diphenylphosphoryl azide; NMM: *N*-methylmorpholine; RGDS: arginyl-glycyl-aspartyl-serine.

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