Transferred-NOE NMR experiments on intact human platelets: receptor-bound conformation of RGD-peptide mimics†

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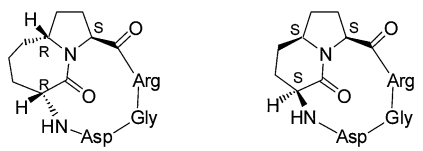
The aim of this work is to show that transferred-NOE provides useful and detailed information on membrane-bound receptor–ligand interactions in living cells. Here, we study the interaction between intact human platelets and some ligands containing the RGD sequence. Conformational properties of the free and bound pentapeptides are reported.

Most of the biomedically relevant proteins are membrane bound; as an example, the integrins are the main cell surface receptors mediating cell adhesion to extracellular matrices.[1] The interaction of peptides with biological membranes is central to a number of biological processes, such as the insertion and folding of peptides in membranes, the rupturing of membranes by toxins, the membrane-mediated mechanism of peptide–receptor interactions. To understand the basis of such interactions, characterization of the altered conformational and dynamic properties of the ligand in the membrane bound form are crucial. In contrast to soluble proteins, there is comparatively less information available about ligand–receptor interactions that occur at membrane surfaces. The biophysical environment of a membrane is considerably different from the isotropic extracellular medium. Therefore, the binding affinity or binding specificity of soluble forms may differ from the properties of the native receptor, which is embedded into a membrane. It is therefore desirable to investigate membrane proteins and their binding specificity directly in living cells. NMR-based methods are quite suitable for detecting at a molecular level, the binding interactions between small ligands and biomolecular targeting and to identify new bioactive substances.[2] Among these, STD,[3] NOE pumping,[4] and trNOE[5] focus on the NMR signals of the ligand and utilize NOE effects between protein and ligand. Of these, only 1D-STD has been used so far on living cells,[6] but trNOE also has the potential to offer easy detection of binding events, additionally producing information on the bound conformation of the ligands. Herein, we show that the interactions between small ligands and membrane-bound proteins can be observed by trNOE spectroscopy directly on whole human platelets, without the necessity for isolating the protein receptor. This approach provides key information on the ligands′ binding mode in the natural environment and allows us to deduce information on the structural requirements of the ligand in the bound state. The potential impact of such an approach on drug-discovery processes may be appreciated by taking into account the great importance of small molecules specifically targeting membrane proteins, in the context of anticancer, antibacterial and antiviral drug research.[7]

The most abundant platelet cell surface glycoprotein is the $\alpha_{IIb}\beta_3$ integrin; this calcium dependent heterodimer is responsible for mediating platelet–platelet and platelet–biomaterial interactions.[8,9] The platelet-specific integrin $\alpha_{IIb}\beta_3$ (like $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins) binds to the Arg-Gly-Asp (RGD) motif as the primary recognition sequence in its ligands and the conformation of the RGD sequence is critical for the specificity of this recognition.[10] Recently, the X-ray structure of $\alpha_{IIb}\beta_3$ receptor with four different ligands was reported.[11] These studies show that the RGD binding site of $\alpha_{IIb}\beta_3$ is indeed larger than the $\alpha_v\beta_3$ receptor, therefore, to obtain a good interaction with the receptor site, the RGD sequence must be in the extended conformation.

In previous studies,[12] we investigated the conformational preferences and the activity of a small library of cyclic RGD pentapeptide mimics cyclo(Arg-Gly-Asp-Lactam). The conformational preferences of these compounds are modulated by the ring-size and by configurational properties of the bicyclic lactam. From this library, we selected the two mimics 1 (ST1646) and 2 (Fig. 1) in order to study their binding with platelet integrin. These compounds showed very different conformational preferences in the free state, moreover compound **1** is a high affinity ligand for the $\alpha_V\beta_3$ integrin (IC_{50} 3 nM) and binds to platelet $\alpha_{IIb}\beta_3$ integrin with a IC_{50} 12 μM, which represents an ideal range to test the trNOE experiment between ligand and integral membrane protein of human platelets.[13] Compound **2** showed a good affinity for $\alpha_V\beta_3$ integrin (154 nM) and its behaviour towards $\alpha_{IIb}\beta_3$ integrin has not been previously determined.



1 ST1646 **2**

Fig. 1 Cyclic RGD pentapeptide mimics cyclo(Arg-Gly-Asp-Lactam) **1** and **2**.

The interaction of **1** and **2** with intact human platelets was studied by NMR. NOESY spectra of the pseudo-peptides were recorded and then trNOE experiments in the presence of living human platelets were performed. Transferred intramolecular NOEs are well established as unique sources of structural information on bound ligand. The technique relies on the size-dependence of the intramolecular NOE, which shows slow NOE build-up with weak positive maxima for free ligands and rapid build-up with strong

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† Electronic supplementary information (ESI) available: NOESY spectra of **1** and **2** in the free and bound state; control experiment with mannose. See DOI: 10.1039/b713036h

negative maxima for the bound state. If dissociation of the ligand occurs quickly enough ($K_d > 10^{-7}$ M), a sufficient percentage of the observable free ligand will retain intense negative trNOE and thus binding of a ligand to a receptor protein can be easily detected by looking at the sign and size of the observed NOEs.

Fig. 2a and Fig. 2b show the tr-NOESY (mt = 0.2 s) spectra of the ligand **1**–platelets and ligand **2**–platelets, respectively. The observed trNOE correlations were large and demonstrated that both pentapeptides **1** and **2** bind to the platelet surface, presumably to integrin $\alpha_{IIb}\beta_3$. For comparison, the NOESY spectra of **1** and **2** in the free state (reported in the ESI†) show cross-peaks that are opposite in sign to those of the diagonal. As a control, mannose was added to a platelet suspension containing compound **2**, and a NOESY spectrum was recorded under the same conditions. The platelet membrane does not contain mannose receptors and, as expected, the NOESY spectrum showed negative cross-peaks (trNOEs) for compound **2** and positive cross-peaks for mannose. This suggests that the interaction observed for **2** is not aspecific, but rather depends on a specific receptor on the platelet membrane.[14] (Spectra are reported in the ESI.†)

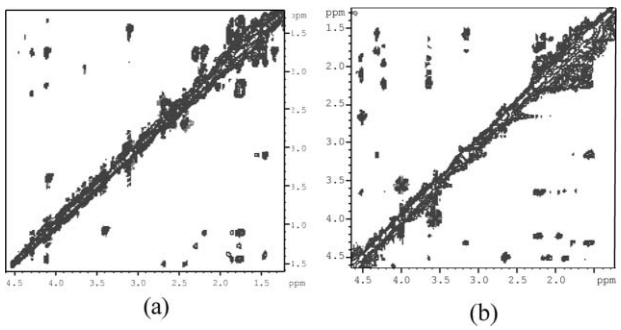

Fig. 2 (a) TrNOESY spectrum (mt = 0.2 s) in D_2O buffer solution of a platelet suspension with **1**. All cross-peaks are in phase with the diagonal (b) TrNOESY spectrum (mt = 0.2 s) in D_2O buffer solution of a platelet suspension with **2**.

Further information can be gained from these experiments about the bound conformation of the ligands also "in comparison" with their conformational behaviour in the free state.

NMR provides a method for the structural description of the peptides and proteins in solution. The methodology generally involves the measurement of a large number of variables such as NOEs and coupling constants followed by a search of the target 3D structure constrained by the same variables. However, observables such as NOEs and chemical shifts reflect averages of the properties of the individual conformations, and the structural characterization of disordered systems cannot be done using conventional static constraints typically used for globular proteins. If the compound is not well-characterized by a single conformation in solution, a virtual conformation may be generated, which combines multi-conformer features in a single geometry. So, we preferred to perform unconstrained computational studies in order to check all the conformational families.

MC/SD simulations (AMBER*, GB/SA water) suggested two geometries mainly contributing to the conformational equilibrium of cyclopeptide **1**.[12] Both these geometries feature almost extended conformations of the RGD sequence (Arg-Cβ–Asp-Cβ = 9.3 Å and 8.6 Å) characterized by a common inverse γ-turn at Asp, and by a distorted βI-turn at Pro-Arg or βII′-turn at Gly-Asp. Energy-minimized conformations of **1** obtained from frames of the 10 ns MC/SD simulation corresponding to these geometries are shown in Fig. 3. Furthermore, compound **1** was fully characterized, by NMR spectroscopy, in D_2O buffer[15] and in H_2O–D_2O solution. The NOESY spectrum of **1** in H_2O–D_2O solution shows NOEs between Gly-NH and Arg-NH (medium) and between Gly-NH and Arg-Hα (medium).[16] These NOE contacts and the chemical shift values of the amide protons are indicative of a β-turn conformation stabilised by a hydrogen bond between Gly-NH and lactamic C=O. Moreover, the chemical shift and the slow exchange rate of lactam-NH are indicative of a hydrogen bonded proton that can form a γ-turn centred on aspartic residue. Other significant strong NOE contacts are between Pro-Hδ–Lact-Hα and between Asp-NH–Gly-$H\alpha_1$ and Asp-NH–Gly-$H\alpha_2$[12] (Fig. 4).

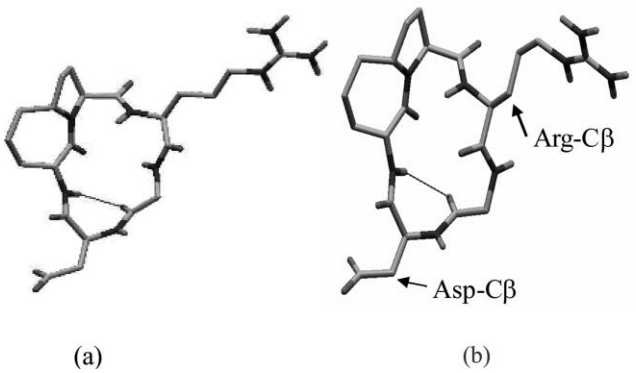

Fig. 3 Conformations of **1** sampled during the 10 ns MC/SD simulation after energy minimization.[12] (a) Inverse γ(Asp)/distorted βI(Pro-Arg) geometry featuring Arg-Cβ–Asp-Cβ = 9.3 Å. (b) Inverse γ (Asp)/distorted βII′(Gly-Asp) geometry featuring Arg-Cβ–Asp-Cβ = 8.6 Å.

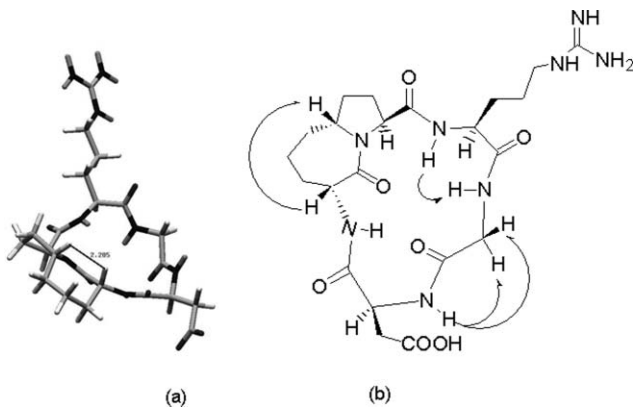

Fig. 4 (a) The same conformation of Fig. 3a is rotated to give evidence to the Pro-Hδ–Lact-Hα interaction. This conformation engaged by the bicyclic moiety forces the RGD backbone geometry and arranges the side chains in an equatorial position. (b) Key NOE connectivities found for compound **1**.

Above all, the Pro-Hδ–Lact-Hα interaction is indicative of a pseudo-boat conformation of the lactam moiety. This conformation engaged by the bicyclic moiety forces the RGD backbone conformation and arranges the side chains in an equatorial position (Fig. 4). The conformation (a) of Fig. 3 is in full agreement with NMR data.

The NOESY spectra performed in deuterated buffer solution[15] and in the presence of platelet suspension showed the same NOE contacts (except NOE contacts relative to amide protons). (Spectra are reported in the ESI.†) For instance, no major change in the NOE contacts between the free and bound state, suggests that the known free state conformation of **1**[12] is conserved upon binding.

For the pentapeptide **2**, MC/SD simulations (AMBER*, H_2O GB/SA) showed different folded conformations in equilibrium.[12]

Fig. 5a presents an energy-minimized conformation of **2** in agreement with NMR data in the free state and corresponding to the most populated geometry of the 10 ns MC/SD trajectory. In this preferred conformation, the lactam ring appears to adopt a half-chair conformation and the cyclopeptide features a γ-turn at Gly and a distorted βII′-turn with Gly at the $i + 1$ position. Compound **2** was characterized, by NMR spectroscopy, in H_2O–D_2O and in D_2O buffer solution.[15] The analysis of chemical shift values of the amide protons suggests that Lact-NH ($\delta = 7.97$ ppm) is involved in an intramolecular hydrogen bond while Gly-NH ($\delta =$ 9.02 ppm) is solvent exposed.[16] The behaviour of Arg-NH ($\delta =$ 6.97 ppm) is typical of a non hydrogen-bound and non solvated proton. The NOESY spectra show the following significant long range cross peaks (Fig. 5b): Lact-NH–Asp-NH (medium), Gly-$H\alpha_1$–Asp-NH (weak), Arg-NH–Pro-$H\gamma_{ax}$ (medium) and Arg-NH–Lact-$H\gamma_{ax}$ (medium). So the amide protons Asp-NH and Lact-NH must be inside the pentapeptide ring and experience a hydrogen bond conctact. Lact-NH binds to the carbonyl group of Arg, stabilizing a slightly distorted β-turn, Asp-NH binds to the same carbonyl group stabilizing a γ-turn at Gly. The NOE contacts of Arg-NH indicate that this amide proton points to the bicyclic scaffold.

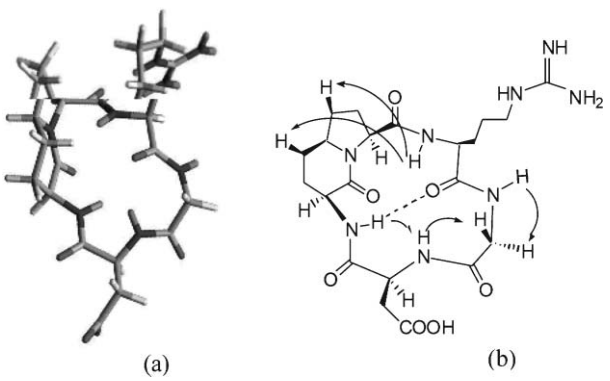

Fig. 5 (a) Preferred conformation of **2** sampled during the 10 ns MC/SD simulation after energy minimization[12] in agreement with spectroscopic data in the free state; in this preferred conformation the lactam ring appears to adopt a half-chair conformation. This γ(Gly)/distorted βII′(Gly-Asp) geometry features Pro-Hδ–Lact-Hα distance = 3.7 Å and Arg-Cβ–Asp-Cβ = 8.2 Å. (b) Significant long range NOE contacts of compound **2** in the free state in H_2O–D_2O solution.

In contrast with the behaviour of compound **1**, trNOE experiments on **2** in platelet suspensions showed a different pattern of cross-peaks compared to the NOESY spectrum in D_2O buffer or in H_2O–D_2O solution and suggested that a conformational change (or a conformational selection process) is taking place upon binding.

In the tr-NOESY experiment of **2** in platelet suspension, we observed a new cross-peak due to the correlation between Pro-Hδ–Lact-Hα (Fig. 6b, in the dotted box); this contact is achievable only if the lactam is forced into a pseudo-boat conformation (Fig. 7). Moreover, different intensities for the Pro-Hα–Pro-$H\beta_1$ and Pro-Hα–Pro-$H\beta_2$ cross-peaks can be detected (Fig. 6a,b, in the circle). These modifications in the bicyclic moiety may correspond to a drastic change in the conformation of the pentapeptide. With the aid of computational studies (from MC/SD simulation) we can select two families of conformations fitting the NOE data of **2** in platelet suspension. In these calculated structures, the distance between Pro-Hδ and Lact-Hα is 2.57 A (Fig. 7a) or 2.56 A (Fig. 7b). In any case, Lact-NH is engaged in a hydrogen bond with the carbonyl group of Gly stabilising a γ-turn at Asp, producing cyclopeptide geometries that differ significantly from the preferred conformation observed in the free state. The rotation of the peptide bond Gly-Asp changes the disposition of the RGD backbone modulating the distance between the side chains of Arg and Asp,

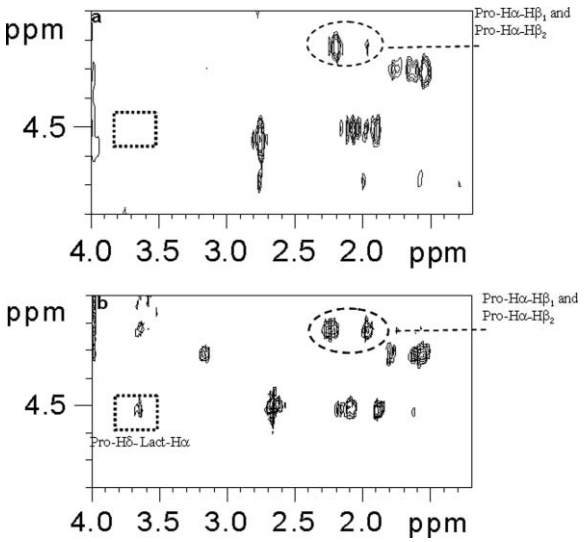

Fig. 6 (a) TrNOESY spectrum (mt = 0.6 s) in D_2O buffer solution of compound **2**. (b) TrNOESY spectrum (mt = 0.2 s) in D_2O buffer solution of a platelet suspension with **2**.

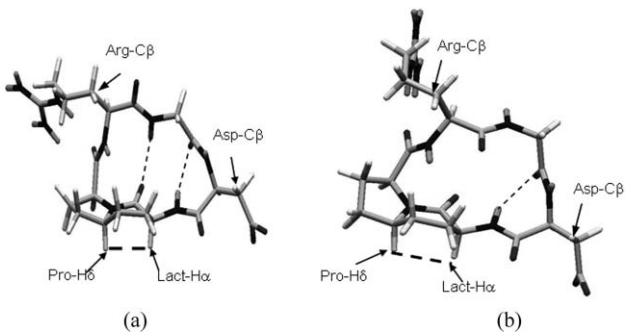

Fig. 7 Conformations of **2** sampled during the 10 ns MC/SD simulation after energy minimization and in agreement with NOE data in platelet suspension. (a) Inverse γ(Asp)/βI(Pro-Arg) geometry featuring Pro-Hδ–Lact-Hα distance = 2.57 Å and Arg-Cβ–Asp-Cβ = 9.4 Å. (b) Inverse γ (Asp)/distorted βII′(Gly-Asp) featuring Pro-Hδ–Lact-Hα distance = 2.56 Å and Arg-Cβ–Asp-Cβ = 8.6 Å.

a key factor in the specificity of ligand binding. Each cyclopeptide geometry shows a specific Cβ(Asp)–Cβ(Arg) distance value that is indicative of the RGD disposition: the conformation of **2** in the free state (Fig. 5a) is characterized by a shorter Cβ(Asp)–Cβ(Arg) distance value (Arg-Cβ–Asp-Cβ = 8.2 Å) and a stronger kink of the RGD sequence in comparison with the bound conformations (Arg-Cβ–Asp-Cβ = 9.4 Å and 8.6 Å, Fig. 7). It is worth noting how the cyclopeptide conformations of **2** in platelet suspension (Fig. 7) coincide with the preferred geometries exhibited by **1** both in the free and the bound state (Fig. 3).

Conclusion

In conclusion, we have successfully carried out ^{1}H transferred NOE experiments on whole human platelets to study small ligand interactions to cell surface proteins in their natural environment. The results of these experiments provide significant insight for the elucidation of the "bioactive" (bound) conformation of the antagonists **1** and **2** to the $\alpha_{IIb}\beta_3$ integrin receptor. The cyclic pentapeptide **2** provides a particularly clear demonstration that the presentation of the RGD motif in peptides and proteins is critical to integrin recognition. The solution structures in the free state show that the presentation of the RGD motif in the two compounds is quite different, explaining their different integrin binding activities. However, both bound structures exhibit a γ-turn centred on Asp residue which plays an important role in modulating the distance between the side chains of Arg and Asp, a key factor in the specificity of ligand binding.

The X-ray structure of the $\alpha_{IIb}\beta_3$ receptor[11] with four different ligands showed that a good binder is obtained when the distance between the acid and basic moieties is longer than in the extended conformation of the RGD sequence. Our NMR results are in agreement with X-ray data.

It could still be argued that because of the large heterogeneity in the possible binding sites, it is not proven by the experiments described above that the observed binding event really involves an integrin receptor. We think that this hypothesis is highly unlikely in view of the fact that the $\alpha_{IIb}\beta_3$ integrin is the most abundant platelet surface protein and in the light of the proven activity of **1** in a selective $\alpha_{IIb}\beta_3$-mediated platelet aggregation assay with a 12 μM IC_{50}.[12] In any case, the results presented here show that binding events at the cell surface can be observed and analyzed by NMR. The tr-NOESY experiments represent a competitive and complementary method to obtain pharmacophore information from the bound conformation of ligand in the natural environment of membrane-bound protein receptors. These results will facilitate the design of novel integrin antagonists and establish the conditions for the NMR studies of the receptor-bound conformations of other integrin antagonists.

Material and methods

Platelets were extracted from human blood received from the Laboratorio Diagnosi Piastrinopatie, Centro Emofilia e Trombosi (Dr Andrea Artoni) of the Ospedale Maggiore of Milan. A portion of 15 mL of CPD plasma (plasma suspended in citrate-phosphate-dextrose solution) was centrifuged and the remaining pellet cautiously suspended in 2 mL of deuterated TRIS-buffered saline containing EDTA (1 mM).[14] This procedure was repeated twice. Four additional washing–centrifuging cycles were performed using deuterated TRIS-buffered saline containing 1 mM $CaCl_2$. After the last centrifugation, the pellets were suspended in 1 mL of the last buffer and the suspension was split into two NMR tubes. To each tube, 1 mg of ligand was added.

NMR experiments were performed at a temperature of 294 K on Bruker Avance 400 MHz and 600 MHz spectrometers. All proton and carbon chemical shifts were assigned unambiguously for **1** and **2** in the free state. The NMR experiments were carried out in a D_2O–H_2O (2 : 8) mixture, in order to observe amide protons, and in D_2O-buffer solution. No change was observed in the ^{1}H and ^{13}C chemical shifts for the different solutions. Initial two-dimensional experiments (TOCSY, COSY, NOESY and HSQC) were carried out on samples of **1** and **2** at sample concentrations of 3 mM. NOESY spectra were performed at 0.2, 0.4, 0.6 and 0.8 s. When necessary, the water resonance was saturated with an excitation sculpting sequence from the Bruker library. The conformation of the two pentapeptides was first analysed with respect to hydrogen bonding of amide protons and NOE contacts. In the bound state, the tr-NOESY experiments were conducted at 0.2 s. The NMR solution stays relatively clear for all the time of acquisition (about 1 hour). We also conducted trROESY experiments to check the spin diffusion effect: no spin diffusion effect was observed. No transferred NOEs were detected in the control experiments for the free ligands at identical conditions without the presence of platelets.

Molecular mechanics calculations using the MacroModel[17] version 7.0 implementation of the Metropolis Monte Carlo/Stochastic Dynamics (MC/SD) simulation algorithm,[18] the Amber all-atom force field[19] (AMBER*) and the implicit water GB/SA solvation model[20] were carried out to investigate the conformational preferences of RGD cyclopeptides **1** and **2**. The simulations were performed at 300 K. A time step of 1 fs was used for the SD part of the algorithm. The total simulation time was 10 ns.

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Notes and references

1 E. Rouslahti, *Annu. Rev. Cell Dev. Biol.*, 1996, **12**, 697–715.
2 B. Meyer and T. Peters, *Angew. Chem., Int. Ed.*, 2003, **42**, 864–890; T. Diercks, M. Coles and H. Kessler, *Curr. Opin. Chem. Biol.*, 2001, **5**, 285–291.
3 M. Mayer and B. Meyer, *Angew. Chem.*, 1999, **111**, 1902–1906; M. Mayer and B. Meyer, *Angew. Chem., Int. Ed.*, 1999, **38**, 1784–1788.
4 A. Chen and M. J. Shapiro, *J. Am. Chem. Soc.*, 2000, **122**, 414–415.
5 B. Meyer, T. Weimar and T. Peters, *Eur. J. Biochem.*, 1997, **246**, 705–709; T. Haselhorst, J. F. Espinosa, J. Jimenez-Barbero, T. Sokolowski, P. Kosma, H. Brade, L. Brade and T. Peters, *Biochemistry*, 1999, **38**, 6449–6459; M. Mayer and B. Meyer, *J. Med. Chem.*, 2000, **43**, 2093–2099; C. B. Post, *Curr. Opin. Struct. Biol.*, 2003, **13**, 581–588.
6 B. Claasen, M. Axmann, R. Meinecke and B. Meyer, *J. Am. Chem. Soc.*, 2005, **127**, 916–919; S. Mari, D. Serrano-Gomez, F. J. Canada, A. L. Corbì and J. Jimenez-Barbero, *Angew. Chem., Int. Ed.*, 2005, **44**, 296–298.
7 T. A. Springer, J. H. Wang, *Cell Surface Receptors*, ed. K. C. Garcia, Elsevier, San Diego, 2004; T. Diercks, M. Coles and H. Kessler, *Curr. Opin. Chem. Biol.*, 2001, **5**, 285–291.
8 M. H. Ginsberg, X. Du, T. E. O'Toole and C. JLoftus, *Thromb. Haemostasis*, 1995, **74**, 352.

9 J. J. Calvete, *Thromb. Haemostasis*, 1994, **72**, 1.
10 E. Rouslahti and M. D. Pierschbacher, *Science*, 1987, **238**, 491–497.
11 T. Xiao, J. A. Tagaci, B. S. Coller, J.-H. Wang and T. A. Springer, *Nature*, 2004, **432**, 59–67.
12 (*a*) L. Belvisi, A. Bernardi, A. Checchia, L. Manzoni, D. Potenza, C. Scolastico, M. Castorina, A. Cupelli, G. Giannini, P. Carminati and C. Pisano, *Org. Lett.*, 2001, 1001–1004; (*b*) L. Belvisi, A. Bernardi, M. Colombo, L. Manzoni, D. Potenza, C. Scolastico, G. Giannini, M. Marcellini, T. Riccioni, M. Castorina, P. LoGiudice and C. Pisano, *Bioorg. Med. Chem.*, 2006, **14**, 169–180; (*c*) L. Belvisi, T. Riccioni, M. Marcellini, L. Vesci, I. Chiarucci, D. Efrati, D. Potenza, C. Scolastico, L. Manzoni, K. Lombardo, M. A. Stasi, A. Orlandi, A. Ciucci, B. Nico, D. Ribatti, G. Giannini, M. Presta, P. Carminati and C. Pisano, *Mol. Cancer Ther.*, 2005, **4**, 1670–1680.
13 The tr-NOESY experiment in general works well for ligands that have K_D values in the range μM to mM.
14 The experiment with a natural competitive binder like fibrinogen cannot be performed in this situation because fibrinogen induces platelet aggregation and precipitation.
15 TRIS-buffered saline was prepared in deuterium oxide(D_2O, 99.9%) and contains 10 mM perdeuterotris(hydroxymethyl)aminomethane (TRIS-d_{11}), 150 mM NaCl, 4 mM NaN_3, 3 mM KCl, 1 mM 4-(2-aminoethyl)benzosulfonylfluoride (AEBSF). All preparations were done at room temperature.
16 The tables of chemical shifts are included in the ESI†.
17 F. Mohamadi, N. G. J. Richards, W. C. Guida, R. Liskamp, M. Lipton, C. Caufield, G. Chang, T. Hendrickson and W. C. Still, *J. Comput. Chem.*, 1990, **11**, 440.
18 F. Guarnieri and W. C. Still, *J. Comput. Chem.*, 1994, **15**, 1302.
19 S. J. Weiner, P. A. Kollman, D. T. Nguyen and D. A. Case, *J. Comput. Chem.*, 1986, **7**, 230.
20 W. C. Still, A. Tempczyk, R. C. Hawley and T. Hendrickson, *J. Am. Chem. Soc.*, 1990, **112**, 6127.

