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Targeting Matrix Metalloproteinases: Design of a Bifunctional Inhibitor for Presentation by Tumour-Associated Galectins

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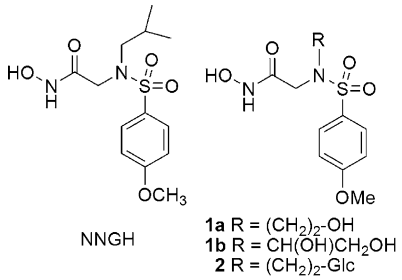
In memory of Prof. Ivano Bertini

Matrix metalloproteinases (MMPs) have long been considered promising therapeutic targets and catch the attention of many researchers but with disappointing results. Nonetheless, increasingly strong evidence for the involvement of MMPs in the development of diverse pathologic states, such as tumour invasion, gives reason to consider these proteases worthy of a renewed interest, though in a new perspective.[1] In this context, we recently reported the rational design and the synthesis of a family of water-soluble MMP inhibitors (MMPIs) **1a**,**b** and **2** that are structurally related to NNGH but featuring a hydrophilic rather than hydrophobic substituent R.[2] Low-nanomolar affinities were reached with these new inhibitors towards therapeutically relevant MMPs, including MMP-9 (Scheme 1).

The main improvement gained by inhibitors **1** and **2** with respect to the large number of reported MMPIs[3] concerns their solubility in water. This is achieved by replacing the isobutyl residue of NNGH with hydrophilic groups. The choice of the hydrophilic residue enables the solubility of the inhibitors to be modulated without affecting their affinity to MMPs. Importantly, this possibility for synthetic flexibility opens the route to address a major issue in targeting MMP activity, namely to interfere with MMPs selectively at sites of disease progression.[4] In principle, installing a hydrophilic group at the sulfonamidic nitrogen, which can account for a preferential presentation of the inhibitor conjugate, for example in or around tumours, would increase therapeutic efficiency and reduce the extent of side effects. Along this line, it was necessary to identify a receptor–ligand pair that would fulfill these two prerequisites; that is, to probe tumour cell or stroma MMPs overexpression and featuring a hydrophilic substituent to direct the conjugate to galectins with sufficient affinity.



Scheme 1. Structure of NNGH and inhibitors **1a**,**b** and **2**.

The far-reaching functionality of endogenous lectins combined with their intriguing selectivity for glycans[5] directed our interest to establish a proof-of-principle for a physiologically relevant test case. Clinical association of expression of galectins to tumour progression[6] attracted our attention. Furthermore, the presence of galectins in the zone of tumour invasion and the role in tumour progression by up-regulating MMP production reveal a functional connection between these two effector proteins and their potential for the suitable positioning of a ligand in situ, even with the added benefit to block clinically unfavourable galectin activities.[7] As the term implies, these adhesion/growth-regulatory lectins show a considerable affinity to β-galactosides so that the introduction of carbohydrates as residue in MMP ligands favourably meets the requirement for the presence of a hydrophilic substituent in sulfonamidic-based inhibitors. In this pilot study, we focus on galectin-3.

This tumour-associated lectin is itself a substrate of MMP-2, MMP-7, MMP-9 and MMP-14.[8] The removal of the col-

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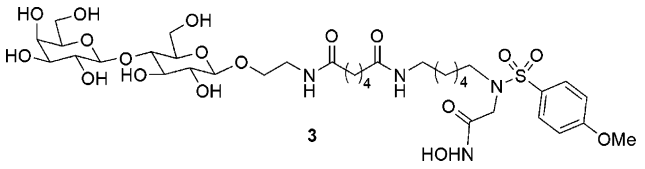
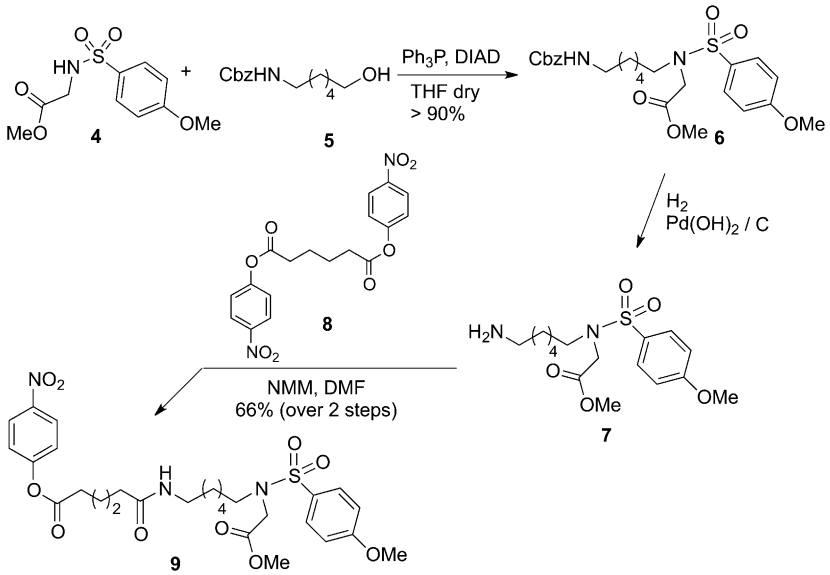
lagenous tail from the lectin domain impairs the capacity for oligomerization in the presence of multivalent ligands;[9] lectin oligoconjugation is known to promote angiogenesis and osteoclastogenesis in breast cancer and bone metastases.[10] By this process, the affinity for lactose is not altered so that both full-length and truncated forms of galectin-3, as well as other tumour-associated galectins, preserve the binding properties versus the ligand.[11] Of note, work with substituted lactosides has revealed that the aglycone extension does not impair ligand activity; conversely, it may even increase the reactivity and introduce inter-galectin selectivity.[12] Having thus explained the selection for lectin type and substituent, we report the synthesis of the bifunctional MMP inhibitor **3**, which is related to **2** and endowed with an assumedly strategic bioactive lactose moiety (Scheme 2). Its ligand properties were analysed using the carbohydrate recognition domain (CRD) of chicken galectin-3 (CG-3),[13] both experimentally by NMR spectroscopy studies and computationally by molecular modelling.



Scheme 2. Structure of ligand **3**.

This new ditopic molecule is considered as lead compound, combining the MMP inhibition property with the galectin-targeting and -blocking capacity owing to a galactoside substituent. Upregulated presence in tumours and also in inflammation (galectin-3 is also known as MAC-2 antigen, a macrophage marker),[14] together with MMP activity on galectin-3, make this lectin family member ideal for testing.

Compound **3** is a sulfonamide presenting a hydroxamic acid as zinc-binding group linked to lactose through an amidic spacer. The length of the spacer was chosen to allow interactions with both the lectin and the catalytic domain of MMP and to prevent the two proteins to clash.[15] The synthesis of compound **3** was performed by a strategy shown in Scheme 3 and 4. The sulfonamide of the glycine methylester **4** was reacted with a benzyloxycarbonyl (Cbz)-protected aminohexanol **5** under Mitsunobu reaction conditions[16] to form methylester **6** in quantitative yield. Removal of the



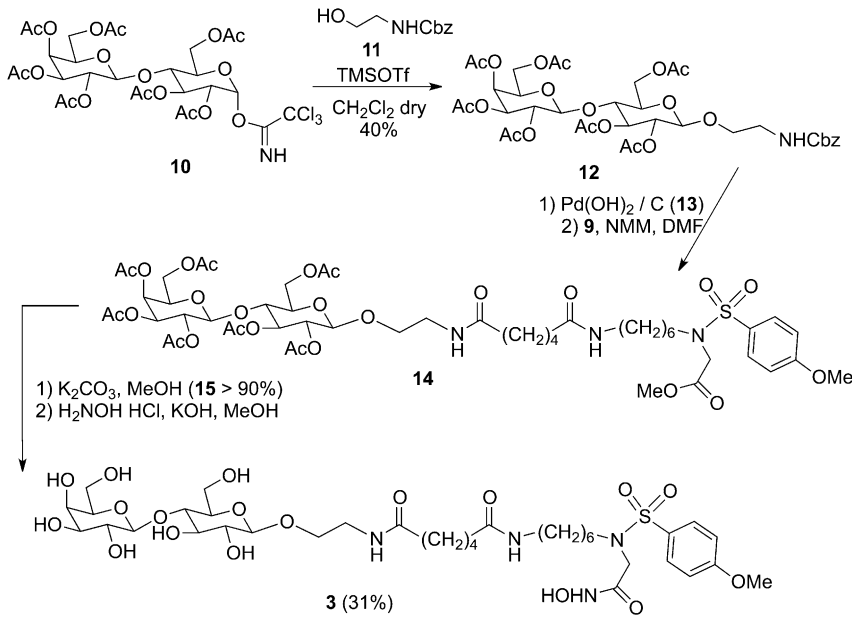
Scheme 3. Synthesis of sulfonamide **9**.

Cbz protecting group (H_2/$Pd(OH)_2$/C) afforded amino derivative **7**, which was treated with adipic ester **8**[17] and *N*-methyl morpholine (NMM) in DMF as solvent to give **9** (66% over two steps; Scheme 3). Glycosyl donor **10**, obtained from peracetylated lactose and trichloroacetonitrile under Schmidt reaction conditions,[18] was first processed with N-Cbz-protected ethanolamine **11** in the presence of trimethylsilyl triflate as promoter to form the β-*O*-glycosyl derivative **12** (40%). After removal of the protecting group (H_2-$Pd(OH)_2$/C) the resulting lactose derivative **13** was reacted with **9** and NMM in DMF as solvent to afford the sulfonamide **14** (90% over two steps).

Deacetylation on the saccharidic portion (K_2CO_3, MeOH) transformed **14** into **15** (>90%). Methyl ester **15** was treated with hydroxylamine hydrochloride and potassium hydroxide in methanol as solvent to generate the desired bifunctional product **3** (31%; Scheme 4). This compound enabled the testing of the assumed dual activity, that is, to inhibit MMPs and to bind galectin.

The inhibitory potency of compound **3** was tested on a panel of six MMPs: MMP-1, 7, 8, 9, 12 and 13 (see the Supporting Information for details). Assays used the catalytic domain of the proteins. As previously observed,[2b] except for MMP-1 and MMP-7, compound **3** showed low-nanomolar K_i values for the MMPs tested. This confirms that the functionalization with lactose did not impair the inhibitory activity of the ligand.[2a]

To investigate the ability of **3** to bind MMP and Gal-3 simultaneously, a series of NMR experiments were carried out, focusing our attention on the ligand (STD and trNOESY) as well as on the proteins (HSQC titration on ^{15}N-labelled proteins). The catalytic domain of MMP-12 (catMMP-12) was chosen as a model, since a full assignment of the respective signals in solution is available and the binding affinity of the sulfonamide scaffold for MMP-12 is



Scheme 4. Synthesis of ligand **3**.

similar to those for gelatinases. As a model for these interaction studies, we used the CRD of CG-3, first studying the binary complexes of both proteins with compound **3**.

For the CG-3↔ligand **3** binary complex, the STD spectrum (Figure 1), recorded under standard conditions (see also the Supporting Information), clearly showed that the

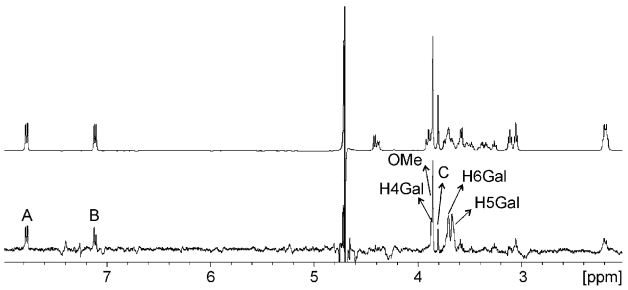


Figure 1. Saturation-transfer difference (STD) spectrum for a sample of CG-3 (100 μM) and compound **3** (5 mM). Top: the off-resonance reference spectrum. Below: the STD spectrum. An STD of CG-3 acquired under the same conditions was subtracted to the STD of the CG-3+ligand sample to remove the protein background signals. The protons of the ligand showing STD are annotated.

galactose residue is the portion of the ligand receiving high-saturation transfer from the lectin, in particular protons H-5 (100%), H-6 (60%) and H-4 (53%). In fact, these protons are prominent when testing free lactose, and also human galectins.[19] Noteworthy, the protons of the methoxyphenyl group also received saturation transfer from the protein, indicating additional carbohydrate-independent binding of this part of the molecule with CG-3.

To the further define structural aspects of CG-3↔ligand **3** recognition, a trNOESY experiment was run on a sample of CG-3/ligand **3** mixture (molar ratio 1:20; see the Supporting Information for details), and compared with the NOESY spectrum of the ligand. As shown in Figure 2, and in contrast with the observations for the free ligand, the cross-peaks of the galactose moiety of **3** were negative in the presence of CG-3. However, the NOEs corresponding to protons of the spacer, the phenylsulfonamide and the hydroxamic acid moieties remained positive in the presence of the lectin. The sign change of the cross-peaks for the lactose part of **3** in the presence of the lectin indicated that a molecular recognition takes place and exclusively involves

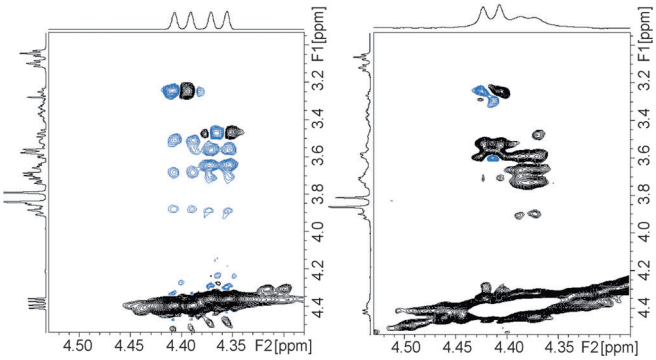


Figure 2. Left: region of the 500 MHz NOESY (500 ms mixing time) spectrum of compound **3** in solution at 298 K. Right: the same region of the 500 MHz trNOESY (250 ms mixing time) spectrum of a sample of CG-3 (100 μM) and compound **3** (2 mM) at 298 K. The regions show the NOE correlations for the anomeric protons: positive (blue) when the ligand is free, and negative (black) in the presence of CG-3.

lactose. Consistently, the simultaneous presence of positive and negative cross-peaks for the different portions of **3** in the presence of the lectin accounts for distinct effective correlation times for each region within **3**. The negative sign for the cross-peaks of lactose reveals that the correlation time for this portion is longer than for those of the spacer or the sulfonamidic substructures. This prolonged correlation time is most likely due to a close contact of the sugar with the protein, as the STD data document, while the rest of the ligand likely retains high flexibility responsible for positive NOEs, even in the presence of CG-3. Apparently, some transient interactions between the lectin and the methoxyphenyl portion might be established, as suggested by the presence of signals in the aromatic region of the STD spectrum (protons A, B and C in Figure 1).

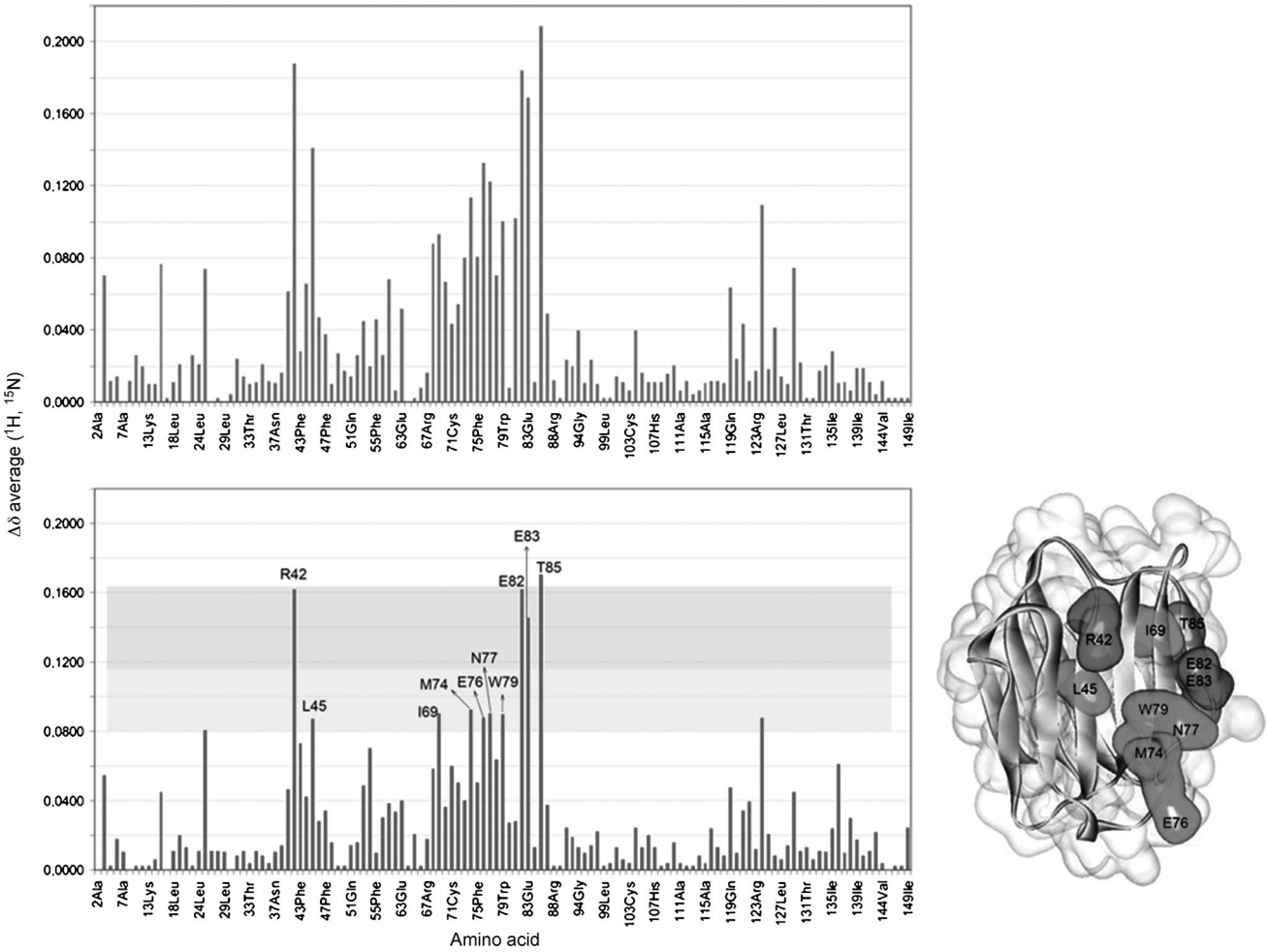


Figure 3. Average chemical shift perturbation ΔδNH,H of ^{1}H and ^{15}N chemical shifts of CG-3 versus the amino acid sequence upon addition of 10 equiv of lactose (top) and **3** (bottom). The 3D model for the CRD for CG-3 (right) shows the amino acids for which the backbone H/N HSQC cross-peaks shifted the most; 10 mM Tris at pH 7.5, 298 K, 600 MHz for all spectra.

The recognition process was next monitored from the perspective of galectin by carrying out HSQC titration experiments of ^{15}N-CG-3 with increasing amounts of ligand **3** (see the Supporting Information for details). The results were compared with those obtained from parallel titration performed with unsubstituted lactose as ligand. Figure 3 shows the average chemical-shift perturbation on ^{1}H and ^{15}N backbone resonances of CG-3 upon addition of lactose (top) and compound **3** (below). It is clear that the sets of perturbed backbone amino acids are very similar in both cases and, furthermore, of comparable extent. This means that ligand **3** binds to CG-3 at the same site as lactose does, leading us to assume that the binding mode of **3** might be very similar to that of lactose, with the galactose ring stacked on top of W79 residue and establishing a network of hydrogen bonds (Supporting Information, Figure S1).

A putative three-dimensional model of the complex formed was deduced by running MD simulations. The starting structure was built by manually docking lactose at the canonical lactose-binding site, as experimentally assessed by the combined STD/trNOESY/HSQC procedure. The stacking of the indolyl ring of W79 with the hydrophobic patch established by galactopyranose H-3, H-4 and H-5 became evident along the simulation and exhibited an average distance between the two rings of 4.9 Å. Glycosidic torsional angles did not vary, indicating stability of the initial exoanomeric-Φ/syn-Ψ conformer. Having herewith revealed and characterized binding of **3** to the galectin, we next ascertained the interaction of **3** with MMP-12.

We thus analysed the **3**↔catMMP-12 binary complex. Taking into account the similar affinity of **3** for MMP-9 (K_i 80 nM) and MMP-12 (K_i 17 nM) and the availability of the full assignment of the NMR signals of the MMP-12 catalytic domain, either free of substrates or complexed with NNGH,[20] the set of experiments to investigate the formation of the compound-**3**↔MMP-12 complex were carried out using the unlabelled and ^{15}N, ^{13}C-labelled catalytic domain of macrophage metalloelastase (MMP-12; see also the Supporting Information).

STD and trNOESY were not helpful to shed light onto the interaction mode between the two partners. Very probably, the strong interaction provided by the aryl sulfonamidic

hydroxamic acid with MMP-12 is responsible for the lack of signals observed in both experiments. Indeed, tight binding causes k_{off} to be very slow. With ^{15}N-labelled MMP-12 (0.1 mM) in Tris buffer (20 mM; see the Supporting Information for details), titrations with a stock solution of **3** in D_2O were performed using different MMP-12/ligand **3** molar ratios: 1:0.2, 1:0.5 and 1:1. As expected, for 1:0.2 and 1:0.5 ratios, two different states of the protein, in slow equilibrium on the chemical-shift timescale, were detected (shown for G178 in Figure 4). These two states correspond to ligand

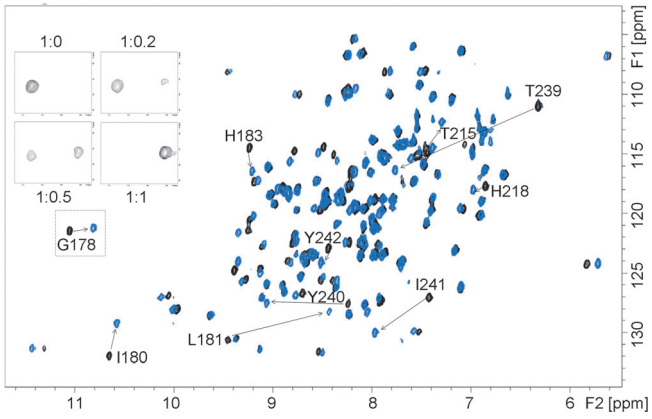


Figure 4. Superimposition of the ^{1}H–^{15}N-labelled HSQC spectra (600 MHz) of 0.1 mM ^{15}N catMMP-12 (black) and upon addition of 0.1 mM of **3** (blue). The arrows indicate some of the largest cross-peak shifts observed and the corresponding amino acid residue. The cross-peak corresponding to residue G178 (in dashed box) is shown at ratios 1:0, 1:0.2, 1:0.5 and 1:1.

3-free and ligand **3**-loaded MMP-12 and indicate that the exchange process is slow on the chemical-shift timescale, as a consequence of the strong binding between both partners. The superimposition of the HSQC spectra of the ^{15}N-labelled catalytic domain of MMP-12 (0.1 mM) and a 1:1 ^{15}N MMP-12 (0.1 mM)/ligand **3** (0.1 mM) mixture allowed the cross-peaks of the protein to be identified that showed the larger chemical-shift perturbation upon ligand **3** binding. As depicted in Figure 4, these cross-peaks correspond to residues G178, I180, L181 located in the β-sheet IV, T215 and H 218 located in the α-helix 2 and T239, Y240, R241 and Y242 situated in the L8 loop, all around the catalytic binding site (Supporting Information, Figure S2). These spectroscopic data clearly indicate that the binding mode of ligand **3** is fairly similar to that previously described for other MMPIs structurally related to NNGH.[2b] Also, they add structural information to the proven inhibitor activity. Together, these data set the stage to test whether a ternary complex is formed.

Before investigating the possibility for formation of the CG-3↔ligand **3**↔catMMP-12 ternary complex, we checked whether a direct interaction between CG-3 and catMMP-12 was possible. In the absence of compound **3**, no chemical-shift perturbations of the HSQC cross-peaks of ^{15}N-labelled CG-3 (0.1 mM in Tris buffer) were observed upon the addition of catMMP-12 (0.1 mM; Supporting Information, Figure S3), allowing any direct interaction between the two proteins to be excluded.

To delineate the bifunctionality of **3**, the HSQC spectra of the ^{15}N-labelled catalytic domain of MMP-12 (0.1 mM) in the presence of an equimolar concentration of ligand **3** was recorded. As already described (see above), MMP-12 is fully loaded under these conditions. Upon addition of CG-3 (0.2 mM), a new HSQC spectrum was recorded. Of particular importance, it presents a dramatic decrease of the intensity of the cross-peaks corresponding to the ^{15}N-labelled MMP-12 amino acid residues located at the α-helix-B and in the adjacent β-strands V, IV, III, II and I. Conversely, the intensity of the peaks corresponding to the amino acids in the α-helix-A maintained their intensity (Figure 5).

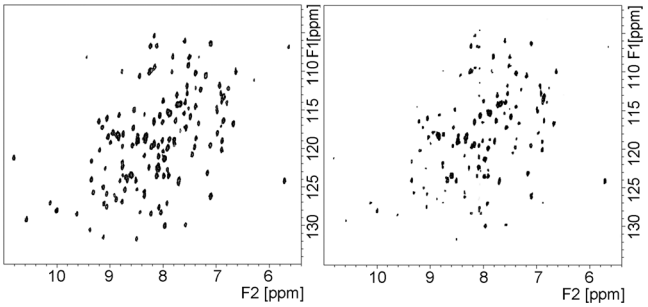


Figure 5. The 600 MHz ^{1}H–^{15}N HSQC spectra. Left: 0.1 mM of ^{15}N-labelled catMMP-12+0.1 mM ligand **3** (ns=64). Right: the same sample after the addition of 0.2 mM of CG-3 (ns=96).

The decrease of the intensity of the signals in the region of the hydroxamate binding site and in the adjacent β-strand strongly suggested that a large molecular weight entity, that is a complex with the lectin, was formed in solution. The exchange process involving the large-size ternary complex should cause a decrease of the effective T2 relaxation time of many residues with the concomitant broadening of the corresponding NMR signals.

Of note, all of the residues belonging to the α-helix-A sited behind the binding site appeared to keep their relaxation properties. In aggregate, these data indicated that the ternary complex was formed in solution and that **3** is able to bind CG-3 and MMP-12 side-by-side.

In summary, we have established compound **3** as a lead substance for the design of effective bifunctional probes, targeting galectins for directing MMPI to specific sites. Clinical documentation of the status of negative prognostic predictors for members of both families, for example, MMP-9/galectin-7 in hypopharyngeal cancer,[7c,21] gives direction to suited in vitro test models. Furthermore, the presence of a galectin network in tumours with functionally antagonistic activities, for example known between galectins-1 and -3,[6,9a,22] can make it necessary to implement selectivity in the sugar part. Appropriate substitutions, along with dendrimeric display, offer ways toward selectivity and affinity increase, as established for the noted case.[23] The formation of

the ternary complex in situ will then not only exploit the lectin for attracting MMPI to the tumour site but also, as an added benefit, block protumoural activities of the lectin at the same time. The same strategy can be applied to other pathologic states with concomitant upregulation of MMPs/galectins, such as rheumatoid arthritis, thus broadening the applicability of this concept. The synthesis of C-lactosides as well as of thioglycosides analogues of compound **3** in dendrimeric display is actually in progress in order to obtain heterobifunctional ligands stable against glycosidases.[12,24]

Experimental Section

Synthesis of compound 14: $Pd(OH)_2/C$ (20% Pd content, 70.0 mg) was added to a stirred solution of **12** (0.150 g, 0.184 mmol) in a mixture of EtOAc/MeOH 3:1 (5.0 mL). The mixture was stirred at RT for 6 h under a H_2 atmosphere then filtered through a pad of Celite. The filtrate was concentrated to dryness to give 0.130 g of crude product **13**, which was used for the next step without further purification. Compound **9** (0.334 g, 0.191 mmol) and NMM (32 μL, 0.287 mmol) were added to a stirred solution of **13** (0.130 mg) in dry DMF (2.0 mL). The mixture was stirred at RT for 6 h then diluted with CH_2Cl_2 (100 mL) and washed with brine (2× 10 mL) and water (2×10 mL). The organic phase was dried over Na_2SO_4 and concentrated to dryness to give 0.270 mg of crude product. The crude product was purified by flash chromatography on silica gel (EtOAc then $CHCl_3$/MeOH 15:1) to give **14** (0.190 g, 90% over two steps) as a yellow oil (see the Supporting Information for details of spectroscopy).

Synthesis of compound 15: K_2CO_3 (0.005 g, 0.036 mmol) was added to a stirred solution of **14** (0.190 g, 0.165 mmol) in MeOH (1.5 mL). The mixture was stirred at RT for 4 h then concentrated to dryness to give 0.145 g of crude product. The crude product was purified by flash column chromatography on silica gel (CH_2Cl_2/MeOH 4:1) to give **15** (0.130 g, 92%) as a white solid (see the Supporting Information for details of spectroscopy).

Synthesis of ligand 3: A suspension of KOH (0.200 g, 3.564 mmol) in MeOH (700 μL) and a suspension of $NH_2OH{\cdot}HCl$ (0.100 g, 1.439 mmol) in MeOH (860 μL) were separately stirred for 10 min at 60 °C. Then 280 μL of the obtained 5.0 M solution of KOH were added to the obtained 1.6 M solution of $NH_2OH{\cdot}HCl$. The reaction mixture was stirred for 10 min at 50 °C, then **15** (0.130 g, 0.152 mmol) was dissolved in 490 μL of the freshly obtained suspension of NH_2OH. The mixture was stirred at RT for 3 h then diluted with MeOH (10 mL) and the solid was filtered off. The filtrate was concentrated to dryness to give 0.123 g of crude product. The crude product was purified by HPLC (column Zorbax 300SB-C18, 9.4×250, 5 um, H_2O/MeOH 60:40 to 10:90) to give **3** (0.040 g, 31%) as a glassy solid (see the Supporting Information for details of spectroscopy).

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Keywords: glycoconjugates · inhibitors · ligand design · metalloproteins · NMR spectroscopy

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