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Characterisation of glycoprotein ligands synthesised using solid-phase combinatorial chemistry

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Abstract

A combination of rational design based on mimicking natural protein–carbohydrate interactions and solid-phase combinatorial chemistry has led to the identification of an affinity ligand which displays selectivity for the mannose moiety of glycoproteins. The ligand was initially identified as 32/18, a triazine scaffold substituted with 2-acetylpyrrole (32) and 5-aminoindan (18). However, characterisation of the immobilised ligand by release from the matrix via a cleavable linker, (4s,5s)-4,5-di(aminomethyl)-2,2-dimethyldioxolane, and using a non-destructive on-resin method, ^{13}C NMR spectroscopy, confirmed that the putative ligand 32/18 was, in fact, 18/18, the disubstituted 5-aminoindan. ^{1}H NMR studies on the interaction of ∝-D-methylmannoside with the ligand 18/18 in solution confirm the involvement of the hydroxyl group in the C-2 position.
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1. Introduction

Affinity chromatography on immobilised lectins has been used extensively to purify glycoproteins [1–4]. However, despite the fact that they have broad group-specificity towards carbohydrate moieties, lectins have yet to find significant application in the commercial production of glycosylated biopharmaceuticals for human administration. This is partly because lectins are biological macromolecules and are thus sensitive to degradation and denaturation by enzymes, detergent treatments and the harsh conditions required to sterilize- or clean-in-place adsorbents, and partly because many lectins are known to be toxic to mammalian cells [5,6]. However, the generic problems faced by biologically-derived ligands may be circumvented by the introduction of “biomimetic” ligands, in which stable synthetic analogues replace natural biological substrates [7,8]. The concept of rational design of durable affinity ligands comprising the generation and screening of focused combinatorial libraries of biomimetic ligands has successfully led to the identification of ligands for the binding and purification of endotoxin [9], immunoglobulin G [10–12], and insulin [13]. More recently, it has been shown that rational design and solid-phase combinatorial chemistry can be applied to develop affinity adsorbents for glycoproteins [14].

A detailed assessment of a number of X-ray crytallographic structures depicting protein–carbohydrate interactions was used to identify key amino acid residues that determine monosaccharide specificity and which were subsequently exploited as the basis for the synthesis of a limited library of glycoprotein binding ligands. One particular ligand (18/18), synthesised on a triazine scaffold with 5-aminoindan (18) as substituent, displayed exceptional binding for the mannoprotein enzyme, glucose oxidase (GOx).

There has been significant interest in the application of NMR spectroscopy for characterising products of solid-phase synthesis and for monitoring the progress of chemical trans-

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formations on resins [15–19]. Since line widths in NMR spectroscopy are greatly influenced by both the mobility of atoms in a sample as well as the homogeneity of the sample matrix, compound immobilisation on solid supports dramatically influences the type and quality of spectral information that can be obtained by NMR spectroscopy. While ^{1}H NMR spectroscopy is sufficiently sensitive to permit rapid analysis of solid-phase reactions, the signals obtained from resin samples by the gel-phase approach are typically too broad (≫25 Hz) to be useful in structure determination. This line broadening can result from the restricted molecular mobility of the tethered compound, as well as from magnetic field inhomogeneity surrounding the sample (susceptibility mismatches at the resin/solvent interface) and homonuclear dipolar interactions. These latter effects can be minimised by rapidly spinning the sample at the "magic angle" with the use of a magic-angle spinning (MAS) probe [20,21]. On the other hand, with gel-phase ^{13}C NMR, the low abundance of the ^{13}C nucleus demands prolonged spectral acquisition times and the sample's spectrum may frequently be confounded by signals resulting from the polymer matrix. Look et al. [22] have described the use of building blocks selectively enriched with ^{13}C which enables the gel-phase method to be employed for convenient rapid reaction monitoring. A similar method was used to characterise ligands synthesised on a ^{13}C enriched matrix.

2. Materials and methods

2.1. Materials

All chemicals were of the highest purity available unless otherwise stated. 2-Acetylpyrrole, cyanuric chloride, epichlorohydrin and 5-aminoindan were from Aldrich (Gillingham, UK). Glucose oxidase and α-D-methylmannoside were obtained from Sigma (London), UK. Sepharose 6B and 6B-CL were purchased from Amersham Biosciences, Uppsala, Sweden while ArgoGel-NH_2 was from Argonaut Technologies (CA, USA).

2.2. Instrumentation

Spectrophotometric readings were taken using a Shimadzu 160A UV–vis recording spectrophotometer. All ^{1}H and ^{13}C NMR spectra were performed on a Jeol JNM Lambda FT NMR spectrometer while mass spectra were recorded on AEI MS50 mass spectrometers, in fast atom bombardment (FAB) mode at the Department of Chemistry, University of Cambridge. Molecular modelling, molecular design and all calculations were performed on a Silicon Graphics (Reading, UK) with a Quanta97 Software package from Molecular Simulations (San Diego, CA, USA).

2.3. Methods

Glucose oxidase activity was followed using the coupled peroxidase-*o*-dianisidine system, while ribonuclease activity was monitored by the decrease in absorbance at 365 nm following hydrolysis of RNA to oligonucleotides [23]. Protein concentration was routinely assayed by the method of Bradford [24] using the Bio-Rad Protein Assay reagent according to the manufacturers protocol. The combinatorial array of ligands synthesised was screened for their ability to bind glucose oxidase, RNase B and RNase A (unglycosylated control) by measuring the absorbance at 280 nm of the unbound and eluted protein. Pure protein standards of glucose oxidase and RNase B/A were used to construct the calibration curves. Primary amines immobilised on agarose beads were detected qualitatively using the ninhydrin spray method. Ninhydrin (0.2%, w/v) in ethanol, was sprayed onto plates containing the aminated agarose and warmed using a hairdryer until a brown to purple coloration was observed. The TNBS method [25] was used to determine amination quantitatively. To 1 g of amine-activated gel washed thoroughly, 500 μl of 5 M HCl was added and heated to 50 °C for 10 min. Upon cooling the hydrolysed gel solution was neutralised with 5 M NaOH, and the final volume of the agarose solution noted. To varying volumes of agarose solution (100–500 μl), appropriately diluted to 1 ml with sodium tetraborate buffer (pH 9.3), 25 μl of 0.03 M TNBS was added. The samples were agitated and allowed to stand for 30 min at room temperature prior to measuring its absorbance at 420 nm. The extent of amine-activation was determined from a calibration curve constructed using 6-aminocaproic acid.

2.4. Combinatorial synthesis of glycoprotein binding ligand library

All ligands designed consist of a *s*-triazine framework with substitution of different groups around the triazine ring. One hundred and fifty milliliters of amine-activated Sepharose 6-CL (24 μmol amino groups/g moist mass gel, 120 g) was thoroughly washed (3 × gel volume), suspended in distilled water (150 ml) and maintained at 0 °C in an ice/salt bath. An ice-cold slurry of cyanuric chloride (14.4 mmol, 2.66 g) dissolved in 40 ml acetone was added in aliquots at 30 min intervals to the gel suspension. The reaction was maintained at pH 7.0–7.5 by adding aliquots of 10% (w/v) NaOH, and kept at 0 °C with continuous stirring for 2 h. The progress of the reaction was monitored by removing small amounts of the coupled gel and performing the qualitative test for primary amines using the ninhydrin spray. Upon completion of coupling cyanuric chloride to the agarose, the gel slurry was washed sequentially with 3 × 10 gel volumes of acetone-distilled water (1:1, 3:1, 1:0, 3:1, 1:1, 0:1 and v/v) to remove unreacted cyanuric chloride and to equilibrate the gel in water. Unless otherwise stated, all substitution of the chlorine atoms in cyanuric chloride with the necessary amines was carried out at amine concentrations five times the original amine concentration on the matrix and equivalent to the cyanuric chloride concentration. The cyanuric chloride-activated gel was divided into five equal portions. R_1 substitution of the chlorine atom was carried out at 30 °C for 24 h with continu-

ous stirring, after which the gel was washed thoroughly with the appropriate solvents, which included acetone, dimethylformamide (DMF), 0.1 M carbonate, pH 10.0 and 0.1 M phosphate buffer, pH 7.0, followed by distilled water.

The gels containing the four different ligands were further sub-divided into five equal portions and R_2 substitution was carried out at 80–90 °C for 72 h in a rotating oven reactor. Upon completion of the ligand synthesis the gels containing the different ligands were washed with the appropriate solvents followed by water and stored in 20% (v/v) ethanol at 4 °C.

2.5. Screening methodology of the glycoprotein ligand library

The glycoprotein ligand library synthesised above were packed into columns (6.2 cm × 0.9 cm; 1 ml) and washed generously with 0.1 M NaOH in 30% (v/v) isopropanol in order to remove any physically adsorbed, i.e. non-covalently attached ligands. This was followed by a water wash and 10 bed volumes of the equilibration buffer, 10 mM Tris, 200 mM NaCl, 20% (v/v) ethylene glycol, 1 mM Mn^{2+} and 1 mM Ca^{2+}, pH 7.0. The appropriate glycoprotein to be screened (GOx; 1 mg/ml, RNase B, 0.1 mg/ml and RNase A, 0.1 mg/ml) dissolved in the equilibration buffer were applied to the columns. The columns were washed with equilibration buffer at a flow rate of 0.4 ml/min and 2 ml fractions were collected until the absorbance at 280 nm reached < 0.01. The bound protein was eluted using 0.5 M α-D-methylmannoside dissolved in the equilibration buffer. The % of protein eluted against total protein loaded and % of unbound protein against protein loaded were used to assess the binding efficiency of the ligands. A column containing unsubstituted cyanuric chloride-activated agarose was used as the control.

2.6. Solid-phase synthesis using a cleavable linker

The compound (4s,5s)-4,5-di(aminomethyl)-2,2-dimethyldioxolane was used as the cleavable linker in the solid-phase synthesis of ligands 32/18, 32/32 and 18/18 (where 32 is 2-acetylpyrrole and 18 is 5-aminoindan) and the controls, agarose-linker and agarose-linker-cyanuric chloride. The linker (0.24 g, 1.5 mmol), in 20 ml acetone:distilled water (1:1, v/v) was added to a 5 ml suspension of epoxy-activated agarose (10 g, 30 μmol/g moist gel mass) in distilled water. Epoxy-activated agarose was prepared in a previously published procedure [21]. The reaction was allowed to proceed in a 30 °C shaker for 12 h and the extent of amination (24 μmol/g moist gel mass) quantified using 2,4,6-trinitrobenzenesulfonic acid (TNBS) method. The linker-substituted agarose (agarose-linker) was washed thoroughly with acetone-distilled water (1:1, 3:1, 1:0, 3:1, 1:1 and 0:1, v/v) to remove unreacted linker and to equilibrate the gel in water. The agarose-linker was used in all further synthesis of the cyanuric chloride-based ligands 32/18, 32/32 and 18/18. The ligands synthesised were packed into columns (0.9 cm × 6.2 cm; 0.5 ml) and screened for their ability to bind GOx. Cleavage of the linker was performed by immersing the linker-attached ligands (2 g) in distilled water (20 ml) and 1 M HCl added to bring the pH to 4–5. The reaction was allowed to proceed at 60 °C for 30 min with continuous agitation. The gel was washed thoroughly with distilled water followed by the addition of 2% (w/v) sodium periodate (5 ml) and left in a rotating oven at 60 °C for 24 h. The cleaved compounds were washed in succession with distilled water, acetone and 0.1 M NaOH in 30% (v/v) isopropanol. The wash solutions were dried in vacuo and left in a desiccator overnight prior to TLC and spectroscopic analysis. The cleaved gels were washed thoroughly with water, packed into columns (0.9 cm × 6.2 cm; 0.5 ml) and re-screened.

2.7. Solid-phase synthesis of ligands on ArgoGel-NH_2 resin and its structural elucidation on gel phase ^{13}C NMR spectroscopy

The ArgoGel-NH_2 resin comprises of a polystyrene backbone lightly (1–2%) cross-linked with divinyl benzene that has been grafted with polyethylene glycol and bears a terminal amine. The resin was used in the synthesis of ligands 32/18, 32/32 and 18/18 and the control, cyanuric chloride-activated ArgoGel. The ArgoGel resin (0.42 mmol/g, 0.2 g) was thoroughly washed (3 × gel volume), suspended in distilled water (10 ml) and maintained at 0 °C in an ice-bath. An ice cold slurry of cyanuric chloride (0.42 mmol, 74 mg) dissolved in acetone (10 ml) was added to the gel suspension. The reaction was maintained at pH 7.0/7.5 by adding aliquots of 10% (w/v) NaOH and kept at 0 °C with continuous stirring for 12 h. The progress of the reaction was monitored by removing small amounts of the coupled gel and performing the qualitative test for aliphatic amines. Upon completion of coupling cyanuric chloride to the ArgoGel resin, the gel slurry was washed sequentially with 3 × 10 gel volumes of acetone-distilled water (1:1, 3:1, 1:0, 3:1, 1:1 and 0:1, v/v) to remove unreacted cyanuric chloride and to equilibrate the gel in water. Further synthesis of ligands 32/18, 32/32 and 18/18 were performed as described above. The gels containing the ligands were washed in acetone and left to dry overnight in a fume cupboard. A sample of the dry ArgoGel resin (150 mg) containing the ligands and the control were placed in separate NMR tubes and [$^2H_6$]benzene (benzene-d6) (800 μl) added. Resin sticking to the wall was coaxed down into the bulk sample by re-wetting with solvent and swinging the tube as though it was a medical thermometer, with care being taken to avoid packing the resin in the bottom of the tube [26]. ^{13}C NMR spectra of the ArgoGel resin attached to putative ligands 32/18, 32/32 and 18/18, cyanuric chloride and pure samples of 32 and 18 were recorded.

2.8. Studies of the interaction between ligand 18/18 and α-D-methylmannoside by ^1H NMR spectroscopy

Solution synthesis of ligand 18/18 (2-(5-aminoindan)-6-(5-aminoindan)-4-chloro-*s*-triazine) was carried out as described previously [27]. The ^1H NMR spectra of α-D-

methylmannoside (10 mg, 50 μmol) and ligand 18/18 (19 mg, 50 μmol) in [$^2H_3$]acetonitrile (acetonitrile-d_3) were taken individually at room temperature on a Jeol JNM Lambda LA400 FT NMR Spectrophotometer. Irradiation of the proton peaks in α-D-methylmannoside was performed to enable assignment of the peaks in this molecule. A few drops of [$^2H_2$]water (water-d_2) was added to the solution of α-D-methylmannoside and mixed thoroughly before the ^{1}H NMR spectrum was retaken. To a fresh solution of α-D-methylmannoside (10 mg, 50 μmol) in [$^2H_3$]acetonitrile (acetonitrile-d_3), the solution of ligand 18/18 was added. The two solutions were mixed thoroughly and the ^{1}H NMR spectrum of the mixture was taken at room temperature. The spectrum of the α-D-methylmannoside:ligand 18/18 mixture was also observed at 60 °C and again at room temperature.

3. Results and discussion

3.1. Determination of the structure of ligand 32/18 using a cleavable linker

The glycoprotein binding ligand (32/18), synthesised on a triazine scaffold with 2-acetyl pyrrole (32) and 5-aminoindan (18) as substituents, displayed exceptional binding for the mannoprotein enzyme, glucose oxidase (GOx), with almost 96% of the adsorbed glycoprotein eluted with 0.5 M α-D-methylmannoside. Further evidence to establish 32/18 as a glycoprotein binder is described by Palanisamy et al. [27]. Once confirmed a glycoprotein binder, the structural characterisation of the 32/18 was carried out using both solid and solution-phase techniques.

Linkers have been commonly used in solid-phase combinatorial chemistry for the characterisation of the synthesised compound [28]. Many linkers are available commercially although the majority of them tend to be aromatic and their attachment and removal are not conducive to use in the relatively fragile agarose-based matrix used for protein adsorption. The compound (4s,5s)-4,5-di(aminomethyl)-2,2-dimethyldioxolane (Fig. 1) was selected as the linker in our studies for a number of reasons: first, the primary amines at both terminals of the compound enables facile attachment to both the matrix and the cyanuric chloride-scaffold. Secondly, it is a small, relatively polar linker, which contains no aromatic structures that might interfere with the ability of the ligand to bind the target protein. Finally, its ease of cleavage using periodate, with minimal disruption of the agarose matrix, makes this an ideal compound to be used as a linker in

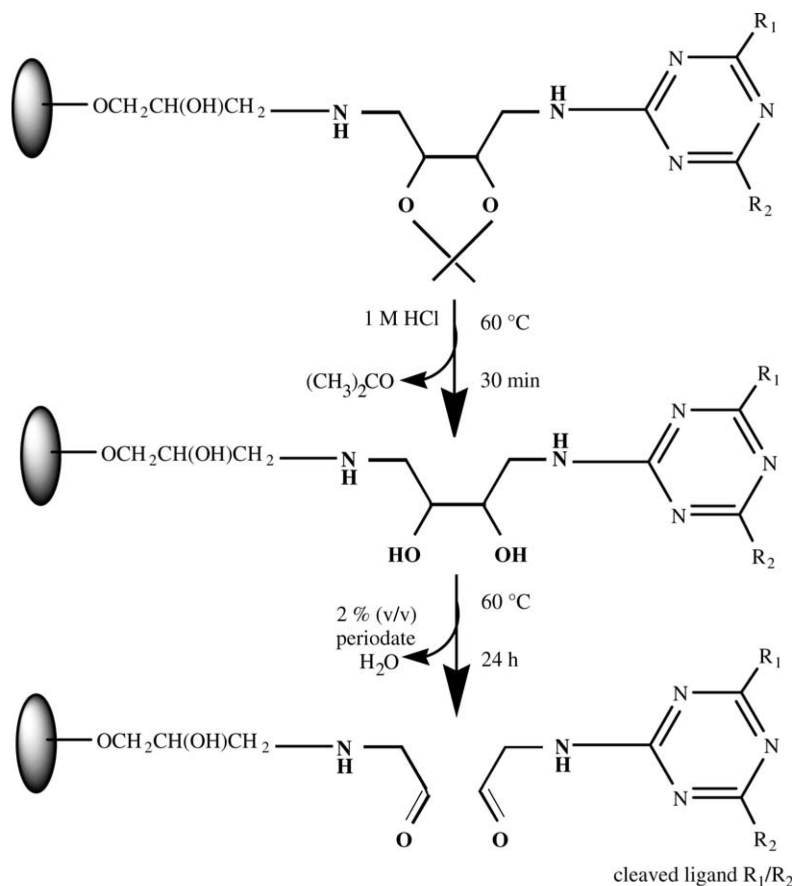

Fig. 1. Ligand attached to the solid support through the linker and the steps involving its cleavage.

Table 1
Ligands screened for their stability to bind and elute GOx before and after periodate cleavage

Ligand	Before cleavage (%)		After cleavage (%)	
	Bound	Eluted	Bound	Eluted
32/18L	96.8	89.4	3.5	0
18/18L	97.8	85.6	2.4	0
32/18[a]	98.2	90.3	97.3	72.1
Agarose-linker	0	0	0	0
Agarose-linker-CC[b]	0	0	0	0

32/18L and 18/18L were synthesised using the cleavable linker where R_1 is 2-acetyl pyrrole and R_2 is 5-aminoindan.
[a] 32/18 was put through the same cleavage conditions as 32/18L and 18/18L.
[b] Cyanuric chloride-activated agarose-linker.

our system. Fig. 1 shows a typical triazine ligand attached to the solid support through the linker and the steps involving its cleavage. The ligands with the cleavable linker, designated 32/18L, 32/32L and 18/18L, and the controls; agarose-linker and cyanuric chloride-activated agarose-linker, were synthesised as described earlier.

These ligands were screened for their ability to bind GOx before and after periodate cleavage (Table 1). The cleaved product(s) were analysed by ^{1}H NMR and mass spectrometry. The ligands 32/18L, 18/18L and 32/18 showed more than 95% binding of GOx with almost quantitative recovery prior to cleavage, while no binding was observed in the controls; agarose-linker and cyanogen chloride-activated agarose-linker. This indicates that the linker does not affect the ability of the ligand to bind GOx. On the other hand, screening after cleavage showed that ligands 32/18L and 18/18L had lost their ability to bind GOx, while 32/18, the ligand without the cleavable linker, still retained its binding ability albeit with a slight reduction in recovery. These observations suggest that the cleavage of the ligand from adsorbents comprising 32/18L and 18/18L had been successful, although periodate may interfere with the quantitative desorption of GOx from the immobilised ligand.

Analysis of the cleaved product(s) of ligands 32/18L and 18/18L by FAB positive mass spectroscopy showed the presence of a mixture of compounds whose structures were unassignable. ^{1}H NMR spectral analysis (Fig. 2) of the cleaved products from adsorbents comprising (a) 32/18L and (b) 18/18L also confirmed the presence of a mixture of compounds and both spectra showed striking similarities. The aryl protons of 5-aminoindan (18), were observed at 8.2, 8.4 and 8.5 ppm, although, most of the other peaks were unidentifiable.

It should be noted that although cleavage of the ligand from its solid support appeared to be successful, several problems were encountered: large amounts (10 g) of the linker-attached ligands were required in order to obtain adequate amounts of the cleaved product for mass spectroscopy or ^{1}H NMR spectral analysis. Furthermore, the cleaved product(s) were a mixture of compounds requiring further purification to identify the compound most active in binding GOx. In order to do this, the cleavage conditions should be optimised, such that a substantial amount of cleaved product(s) is obtained. However, the ^{1}H NMR spectral analysis of cleaved products of 32/18L and 18/18L were very similar with a definite identification of substituent 18 in both, suggesting that they might be one and the same ligand.

3.2. *Determining the structure of 32/18 by gel phase ^{13}C NMR spectroscopy using ArgoGel resin*

The ArgoGel resin based on a polyethylene glycol-polystyrene graft co-polymer was used in this study.

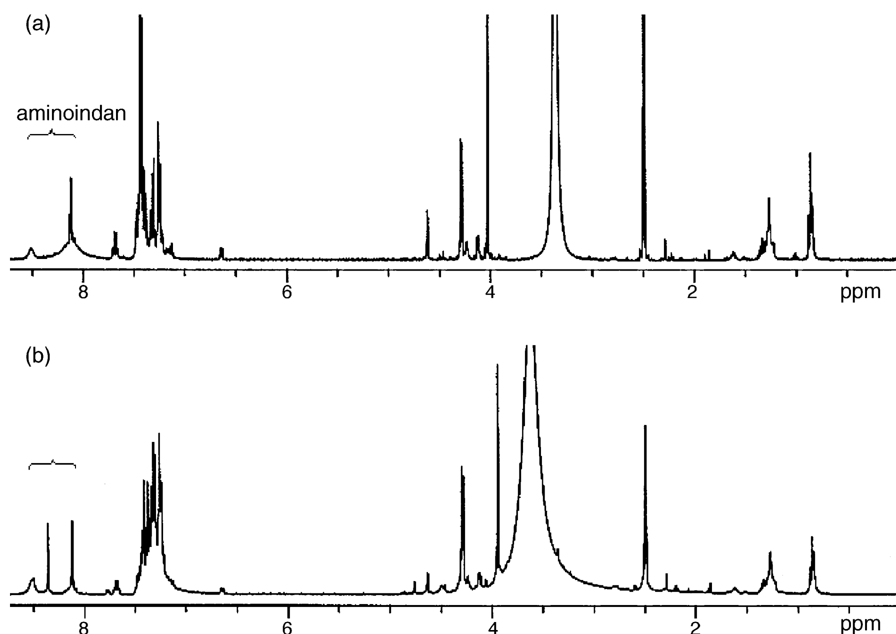

Fig. 2. The ^{1}H NMR spectra of cleaved products of (a) 32/18 and (b) 18/18 in [$^2H_6$]acetone (acetone-d_6).

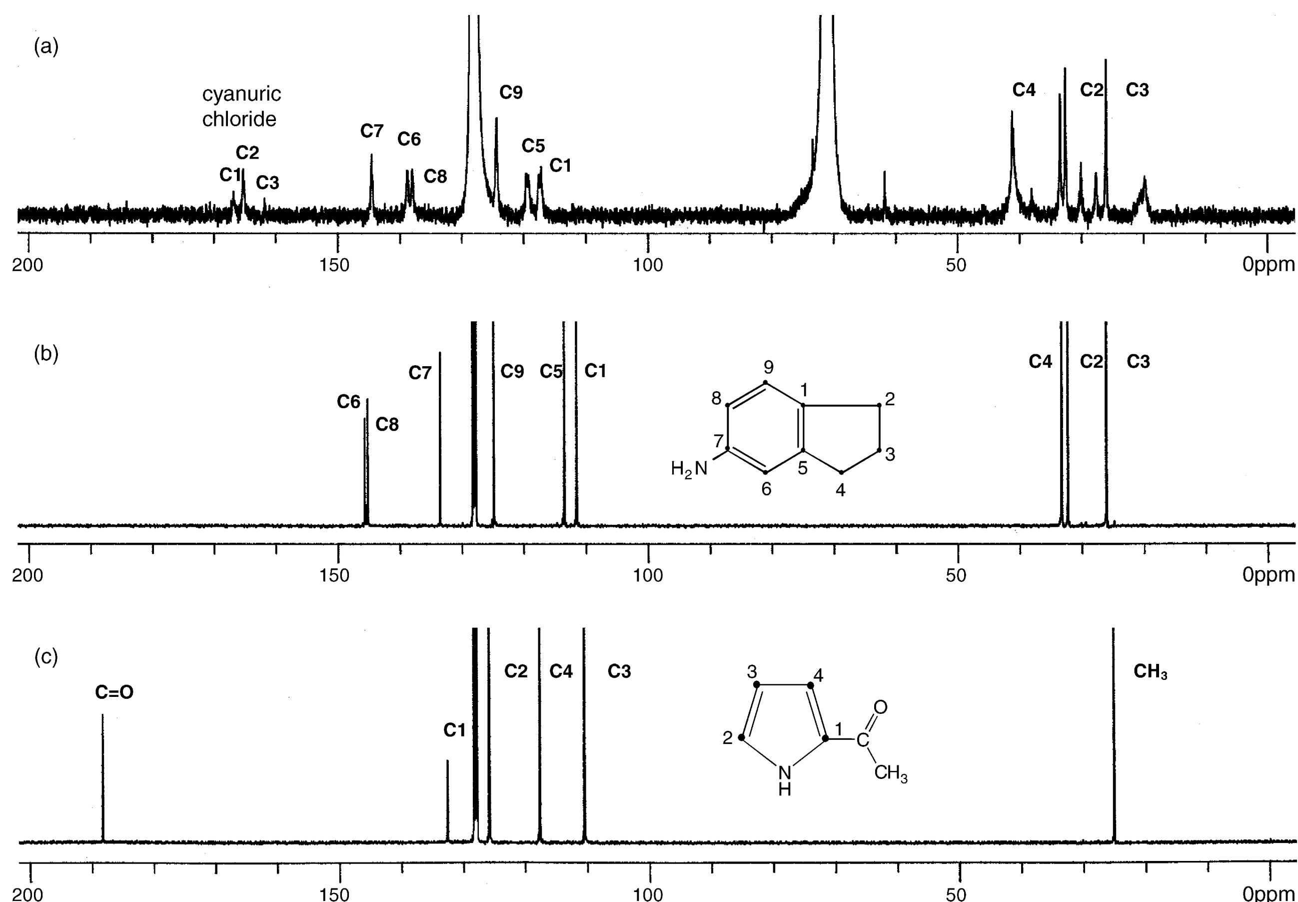

Fig. 3. ^{13}C NMR spectra of (a) ligand 32/18 synthesised on ArgoGel resin (b) 5-aminoindan (18) and (c) 2-acetylpyrrole (32). The spectra was taken in benzene-d_6 at room temperature.

Gel phase ^{13}C NMR on the ArgoGel resin represents a powerful means of characterising synthetic transformations on the solid support. The polyoxyethylene (POE) chain and molecules bound to them are more mobile than the polystyrene backbone units and consequently give relatively sharp NMR lines. Signals from the polyoxyethylene backbone are largely absent due to anisotropic broadening.

The ArgoGel resin was used to synthesise the ligands 32/18, 32/32, 18/18 and the control cyanogen chloride-activated ArgoGel. Gel-phase ^{13}C NMR spectra of ligand 32/18 and standards 2-acetyl pyrrole (32) and 5-aminoindan (18) were taken (Fig. 3). The characteristic peaks of the carbonyl (188.22 ppm) and methyl groups (25.14 ppm) in 32 were not seen in ligand 32/18. However, all the carbon peaks for 18 and cyanuric chloride were observed in the ^{13}C NMR spectra of 32/18. It should also be mentioned that the spectrum for 32/18 was almost identical with that obtained for ligand 18/18 (results not shown). Furthermore, the spectra for the control ArgoGel and 32/32 (results not shown) were also identical indicating that the compound 32 was not substituted onto the triazine scaffold. In addition to the above, solution synthesis (results not shown) of 32-triazine and 18-triazine adducts showed that the nucleophilic substitution of 5-aminoindan (18) with the triazine occurred but not with 2-acetyl pyrrole (32). An explanation for this could be due to pyrroles polymerizing under acidic conditions [29]. Cyanuric chloride (2,4,6-trichloro-*s*-triazine) has been known to hydrolyse at room temperatures produces cyanuric acid [30]. This creates an ideal environment for the pyrrole to undergo polymerization and not react with the triazine.

3.3. Studies of the interaction between ligand 18/18 and α-methylmannoside by ^1H NMR spectroscopy

The nature of the interaction between ligand 18/18 and α-methylmannoside was studied using ^1H NMR spectroscopy. The ^1H NMR spectra of (a) ligand 18/18, (b) α-methylmannoside and (c) the ligand:α-methylmannoside complex is shown in Fig. 4. Proton peaks in α-methylmannoside (Fig. 4b) were assigned after irradiating the individual peaks on the mannoside, while the hydroxyl clusters at 3.06–3.13 ppm (three hydroxyls) and 2.70–2.73 ppm (CH_2OH) were identified on adding deuterated water ($^2H_20$) which led to the formation of hydrogen bonds and thus saw the disappearance of these groups. The disappearance of all three hydroxyl groups (3.06–3.13 ppm) on the mannoside in the ligand 18/18:α-methylmannoside complex (Fig. 4c)

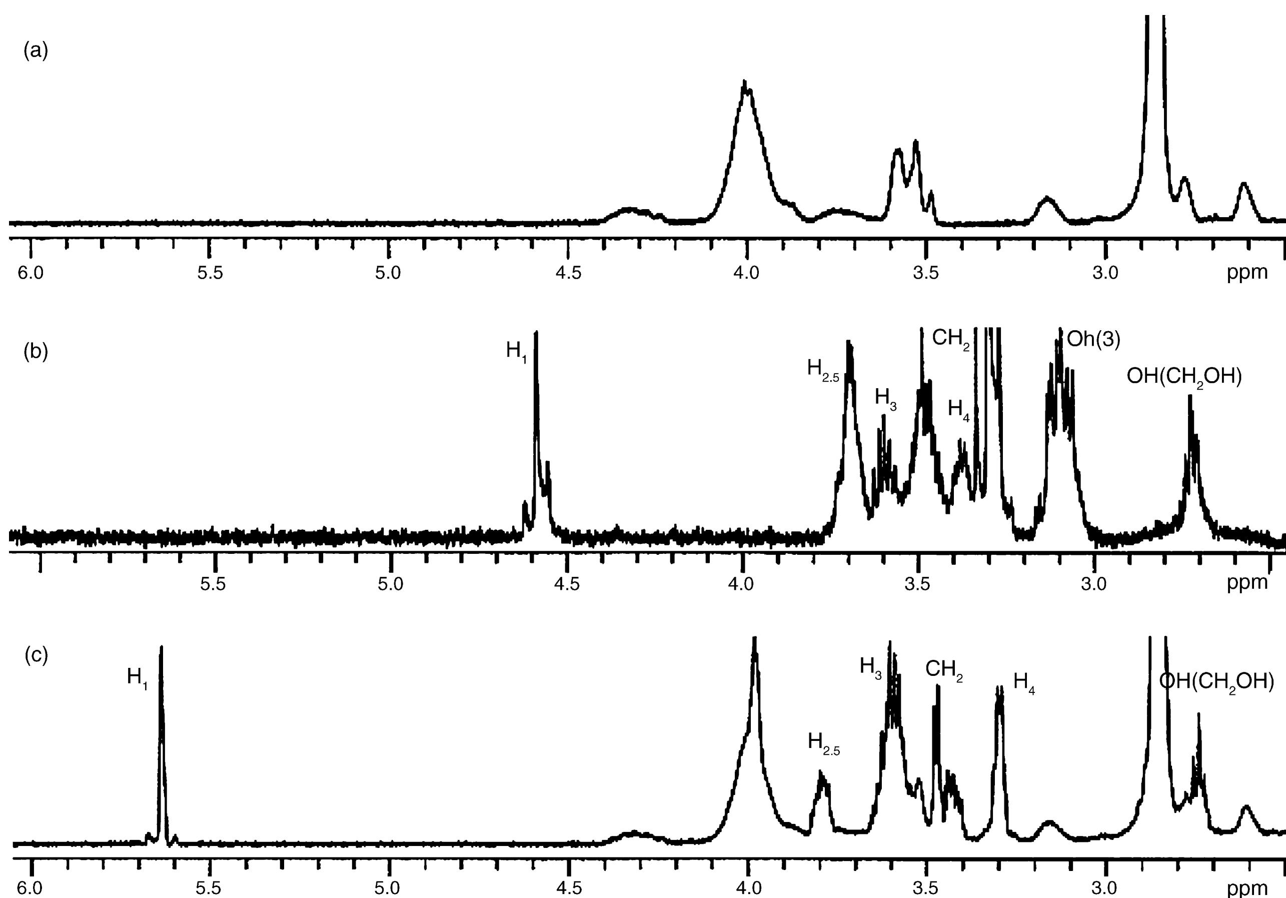

Fig. 4. ^{1}H NMR spectra of (a) ligand 18/18, (b) α-D-methylmannoside and (c) ligand 18/18:α-D-methylmannoside complex in acetonitrile-d_3. The ^{1}H NMR spectra of ligand 18/18, α-D-methylmannoside and ligand 18/18:α-D-methylmannoside complex in acetonitrile-d_6 at room temperature were recorded on a Jeol JNM Lambda LA400FT NMR spectrometer.

strongly suggests the formation of hydrogen bonds with the ligand 18/18.

Furthermore, shifts in the H_1 (4.59–5.63 ppm) and H_2 and H_3 (3.71–3.80 ppm) on the mannoside indicates the involvement of the hydroxyl group at the C-2 position, which has a tendency to affect these protons sterically. It has been pointed out that hydrogen bonds confer specificity and affinity upon carbohydrate–protein interactions and carbohydrate binding proteins [31–34].

To ensure that the disappearance of the hydroxyl groups is in fact due to the formation of hydrogen bonds, the ligand 18/18:α-methylmannoside complex was heated to 60 °C, after which it was allowed to cool down to room temperature again. Upon heating the complex, the weak hydrogen bond interactions will be broken and thus with it the appearance of hydroxyl clusters, and on cooling, these hydroxyl groups were seen to disappear again, confirming the formation of hydrogen bonds between 18/18 and α-methylmannoside. In an earlier paper [27], molecular modelling was performed to support the above evidence on the nature of interaction between the ligand 18/18 and α-D-methylmannoside. Aromatic stacking of the ligand 18/18 was observed against the face of the methyl group of the sugar molecule creating a hydrophobic patch. Similar stacking of aromatic residues against the faces of sugars have been observed in almost all protein–carbohydrate complexes and is said to confer specificity and stability to these complexes [35,36].

4. Conclusions

The ligand initially thought to be 32/18 and synthesised using solid-phase combinatorial chemistry was identified as a putative glycoprotein binding ligand. However, characterisation using NMR spectroscopy strongly suggests that ligand 32/18 was, in fact, the symmetrical ligand 18/18. A cleavable linker designed to characterise the synthesised ligand upon cleavage was identified. Analysis of the cleaved products of ligands 32/18 and 18/18 also confirmed that the ligand was in fact 18/18. Finally, a non-destructive method of on-resin ^{13}C NMR spectroscopy confirmed that the putative ligand 32/18 was indeed 18/18. These results therefore confirm conclusively that the ligand 32/18 synthesised on the solid support is actually the bis-substituted ligand, 18/18. Ligand 18/18 was successfully synthesised in solution and characterised by TLC, ^{1}H NMR spectroscopy and mass spectroscopy. The

final product was obtained in a yield of >96% and was determined to be pure by ^{1}H NMR integration. The ligand was immobilised onto an amine-derivatised agarose matrix and was found to behave in a similar fashion to the ligand (32/18) synthesised using the solid-phase assembly method.

In addition, studies on the nature of interaction between ligand 18/18 and α-D-methylmannoside using ^{1}H NMR spectroscopy suggest the formation of hydrogen bonds, with a strong indication that the hydroxy group at the C-2 position are involved. Hydrogen bonds have in fact been implicated as the major contributors of carbohydrate specificity in protein–carbohydrate interactions of carbohydrate binding proteins [33,34,37].

It is interesting to note that the triazine-scaffold plays a major part in the monosaccharide recognition. However, the fact that the triazine nucleus alone is not a good ligand suggests that both the 5-aminoindan (18) molecules and the triazine are required to be present in a proper conformation for binding the saccharide. An interesting feature revealed in these studies is the similarity in the binding characteristics of ligand 18/18 and the plant lectin concanavalin A (Con A). Both ligands show the involvement of hydroxy groups at the C-2 and C-4 positions. Therefore, it is proposed that ligand 18/18 has great potential to be used in a similar capacity as Con A. In addition, ligand 18/18 has several advantages over Con A, since it is a synthetic ligand and is therefore robust and able to withstand harsh conditions of sterilisation and regeneration, is easy and inexpensive to produce, does not require metal ions for its binding and is immune to biological preparations. However, further studies will be required in order to establish that ligand 18/18 may be used for similar purposes as Con A. This work has clearly demonstrated the power of solid-phase combinatorial techniques for the development of a new affinity ligand for the potential purification of mannose-containing glycoproteins.

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