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Synthesis and evaluation of affinity adsorbents for glycoproteins: an artificial lectin

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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, denoted 18/18 and comprising a triazine scaffold bis-substituted with 5-aminoindan, has been synthesised in solution, characterised by TLC, ^{1}H-NMR and MS. When immobilised to amine-derivatised agarose at concentrations >24 μmol/g moist weight gel, ligand 18/18 selectively binds glucose oxidase. The adsorbed enzyme was quantitatively eluted with 0.5 *M* α-D-methyl-mannoside and to a lesser extent with the equivalent glucoside. An investigation of the comparative retention times of saccharidic solutes showed that significant retardation was observed for α-D-mannose, mannobiose and mannan, with little or no evidence for selective retention of other saccharides, with the exception of α-L-fucose. Interestingly, α-L-fucose and α-D-mannose share an identical configuration of the hydroxyl groups on C-2, C-3 and C-4. Analysis of Scatchard plots from partition equilibrium studies on the interaction of glucose oxidase and the *p*-nitrophenyl-glycosides of D-mannose, D-glucose, L-fucose and D-galactose with immobilised 18/18 establish that the affinity constants (K_{AX}) for the enzyme, the glycosides of mannose, glucose and fucose, and the *p*-nitrophenyl-galactoside are $4.3\times10^5\ M^{-1}$, $1.9\times10^4\ M^{-1}$ and $1.2\times10^4\ M^{-1}$ respectively. ^{1}H-NMR studies on the interaction of α-D-methyl-mannoside with ligand 18/18 in solution confirm the involvement of the hydroxyl group in the C-2 position. Molecular modelling suggests the formation of four hydrogen bonds between the hydroxyl groups at positions C-2, C-3 and C-4 of α-D-methyl-mannoside and the bridging and ring nitrogen atoms of the triazine scaffold, with aromatic stacking of a second ligand against the carbohydrate face. The greater specificity of ligand 18/18 for mannose and glucose than for galactose parallels that exhibited by concanavalin A. © 2000 Published by Elsevier Science B.V.

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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, lec-

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tins 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]. However, the generic problems faced by biologically-derived ligands may be circumvented by the introduction of 'biomimetic' ligands, in which ultra-stable synthetic analogues replace natural biological substrates [6]. Introduction of the concept of rational design of durable affinity ligands comprising the generation and screening of focused combinatorial libraries of biomimetic ligands has sucessfully led to the identification of ligands for the binding and purification of endotoxin [7], immunoglobulin G [8–10] and insulin [11]. The aim of the present work is to design and synthesise biomimetic affinity adsorbents ultimately capable of resolving the multiple glycoforms of pharmaceutical proteins. The ligand should be stable, non-toxic, inexpensive, easily produced, have no cleavable bonds and be able to withstand the harsh conditions required for the in situ sanitization and regeneration of the adsorbents.

Preliminary studies have shown that rational design and solid-phase combinatorial chemistry can be applied to develop affinity adsorbents for glycoproteins [12]. A detailed assessment of a number of X-ray crystallographic 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 (8/10), comprising histamine (8) and tryptamine (10) substituents on a triazine scaffold, was identified as a putative glycoprotein binding ligand, since it displayed particular affinity for glucose oxidase and other mannosylated glycoproteins [12]. Control studies with deglycosylated glucose oxidase, specific sugar eluants and diminished retardation in the presence of competing sugars strongly suggested that the ligand bound to the carbohydrate moiety of the enzyme rather than the protein per se.

It is known, however, that solid-phase reactions give rise to mixtures of products where more than one functionality is present on the reactant [13–15]. Furthermore, both primary and secondary amino functions on histamine (8) and tryptamine (10) would be expected to be reactive towards 2,4,6-trichloro-*sym*-triazine, and hence the defined synthesis of ligand 8/10 in solution would require the use of ring-protected reagents followed by subsequent deprotection and characterisation of the heterobifunctional ligand. In this study, analogues of histamine and tryptamine were investigated in order to find a suitable replacement for the glycoprotein-binding ligand 8/10 which could be synthesised easily on a large scale in solution, fully characterised and immobilised to generate an active glycoprotein-binding affinity adsorbent.

This report describes the solid-phase combinatorial synthesis of a second generation library of 40 analogues of histamine and tryptamine, assessment of their ability to bind glycoproteins and the identification of ligands substituted with the compound 5-aminoindan (18) as good binders. The homobifunctional ligand 18/18 was synthesised in solution, fully characterised by TLC, ^{1}H-NMR and mass spectroscopy and attached to an agarose support via various spacer arms at several ligand densities. The operational conditions required for the satisfactory adsorption and elution of the mannoprotein enzyme, glucose oxidase, have been investigated.

2. Experimental section

2.1. Materials

All chemicals were of the highest purity available unless otherwise stated. 2-Aminoimidazole, pyrrole, imidazole, 2-acetyl-pyrrole, 2-(2-aminoethyl)-1-methyl pyrrolidine, 2-(2-aminoethyl)-pyrrolidine, phenylalanine, 2-(2-aminoethyl)-pyridine, 5-aminoindan, 3-acetyl-indole, 2-aminobenzimidazole, tryptophan, 2-aminopyridine, 4-aminopyridine, tyramine, 1-aminoindan, 2-aminoindan, 4-amino-2,1,3-benzo-thiadazol and 6-aminoindazole were from Aldrich Chemical Co. Ltd., Gillingham, Kent, UK. Glucose oxidase (*Aspergillus niger*; Type X-S), ribonuclease A and B, *N*-acetyl-glucosamine, α-D-

methyl-glucoside, α-D-methyl-galactoside, α-D-methyl-mannoside, α-D-mannose, α-D-glucose, the *p*-nitrophenyl α-D-glycosides of mannose, glucose and galactose, and *p*-nitrophenyl-L-fucoside were purchased from Sigma (London) Chemical Co., Poole, UK. Pre-packed Sephadex G-25 (PD-10 columns) and Sepharose 6B-CL were obtained from Pharmacia Biotechnology, Uppsala, Sweden.

2.2. Preparation and assay of enzyme solutions

Glucose oxidase and ribonuclease A and B activities, protein and ninhydrin assays were performed according to Ref. [12]. Enzyme solutions were prepared by direct dissolution of the freeze-dried preparation in Tris-chloride buffer (20 m*M* Tris–HCl–0.2 *M* NaCl–1 m*M* $CaCl_2$–1 m*M* $MnCl_2$), pH 7.0; and their concentration determined spectrophotometrically on the basis of an absorption coefficient ($A_{1\ cm}$) of 16.0 at 280 nm [16]. A value of 160 000 was used for the molecular weight of glucose oxidase [17] in the conversion of concentrations to a molar basis. Concentrations of the four nitrophenylglycosides were based on a molar extinction coefficient of 8200 (±200) M^{-1} cm^{-1} at 305 nm — the average value obtained by measuring the concentration of nitrophenol in neutralized acid hydrolysates of solutions with known absorbances. Concentrations of methylmannoside were based on the weight of material dissolved in the Tris-chloride buffer, pH 7.0.

2.3. Solid phase combinatorial synthesis and assessment of a second generation glycoprotein-binding ligand library

Lead optimisation of ligand 8/10 was performed by constructing a solid-phase library comprising analogues of histamine and tryptamine on the triazine scaffold according to a previously published procedure [12]. The library contained combinations of the following substituents: 2-aminoimidazole (17), pyrrole (30), imidazole (31), 2-acetyl-pyrrole (32), 2-(2-aminoethyl)-1-methyl pyrrolidine (33), 2-(2-aminoethyl)-pyrrolidine (34), phenylalanine (14), 2-(2-aminoethyl)-pyridine (16), 5-aminoindan (18), 3-acetyl-indole (19), 2-aminobenzimidazole (20), tryptophan (22), 2-aminopyridine (23), 4-aminopyridine (24), tyramine (25), 1-aminoindan (26), 2-aminoindan (27), 4-amino-2,1,3-benzo-thiadazol (28) and 6-aminoindazole (29). Adsorbents from the triazine ligand library synthesised on a solid support (25 μmol/g moist weight gel) were packed into columns (0.9×6.2 cm; 1 ml total volume), washed exhaustively with 0.1 *M* NaOH in 30% (v/v) isopropanol and pre-equilibrated with a buffer comprising 10 m*M* Tris–HCl containing 0.2 *M* NaCl, 20% (v/v) ethylene glycol, 1 m*M* Ca^{2+} and 1 m*M* Mn^{2+}, pH 7.0. Glucose oxidase (GOx^1; 1 mg/ml; 1 ml) was dissolved in the equilibration buffer, applied to the columns at 20°C and the columns washed with the same buffer at a flow-rate of 0.4 ml/min until the absorbance at 280 nm reached zero. The bound protein was eluted with 0.5 *M* α-D-methyl-mannoside dissolved in the equilibration buffer. Dependences of the percentages of protein eluted and unbound protein upon the amount of protein loaded were used to assess the binding efficiency of the immobilised ligands.

2.4. Solution synthesis of ligand 18/18 (2-(5-aminoindan)-6-(5-aminoindan)-4-chloro-s-triazine)

A solution of 5-aminoindan (0.43 g, 3.3 mmol) and $NaHCO_3$ (0.23 g, 2.72 mmol) in 30 ml acetone:cold deionised water (1:1, v/v) was added dropwise to a stirred solution of triazine (0.50 g, 2.7 mmol) in acetone (5 ml) maintained in an ice bath. The reaction was followed by TLC (solvent system; hexane:ethylacetate, 6:1, v/v) until all of the triazine was consumed (5 min). The product was precipitated by adding cold deionised water (10 ml) and the precipitate washed on a sintered funnel with copious amounts of cold deionised water to remove the remaining 5-aminoindan and salt. The product was filtered over a double fluted filter paper and acetone removed in vacuo. Purity of the compound was checked by TLC and its authenticity determined by ^{1}H-NMR, mass spectrometry and melting point.

A solution of 2-(5-aminoindan)-4,6-dichloro-*s*-triazine (0.50 g, 1.8 mmol) in acetone (10 ml) was added to a stirred solution of 5-aminoindan (0.29 g, 2.2 mmol) and $NaHCO_3$ (0.15 g, 1.8 mmol) in 20 ml acetone:deionised water (1:1, v/v) at room temperature. The reaction was followed by TLC (solvent system; hexane:ethylacetate, 6:1, v/v) until all 2-(5-

aminoindan)-4,6-dichloro-*s*-triazine was consumed (15 h). The reaction mixture was concentrated to dryness in vacuo and the residue washed thoroughly with copious amounts of water and dried prior to determining its melting point, purity and authenticity by TLC, ^{1}H-NMR and mass spectrometry.

2.5. Immobilisation of ligand 18/18 to amino-substituted agarose

Sepharose CL-6B was activated with epichlorohydrin for 24 h, samples of the gel being removed at various stages of the incubation to achieve a range of levels of epoxy activation. After removal of the excess epichlorohydrin by extensive washing with distilled water, the gels were analyzed for epoxy content [18]. An 0.880 ammonia solution was then added to the various epoxy-activated gels, after which the aminated gel was washed thoroughly with distilled water. Epoxy-activated Sepharose CL-6B (30 μmol epoxy group per g moist-weight) was also aminated with 1,2-diaminoethane, 1,3-diaminopropane, 1,4-diaminobutane, 1,5-diaminopentane and 1,6-diaminohexane in readiness for the generation of affinity matrixes with various spacer arms.

A 5-fold molar excess of ligand 18/18 over the epoxy content of each gel was then added to the various amino-substituted Sepharose CL-6B gels to provide affinity matrices with varying densities of immobilized ligand 18/18 as well as with various spacer arms. These affinity matrices were packed into columns and screened as described previously [12].

The distance of the ligand from the matrix backbone, taken as the distance from the centre of the first carbon nearest the matrix backbone to the centre of the last nitrogen adjacent to the ligand, was calculated on energy minimised models drawn using CS Chem 3D Pro and CS MOPAC Pro Software (Cambridge Soft Corporation, MA, USA).

2.6. Chromatography of sugars on immobilised 18/18-agarose

Columns (0.9×1.6 cm) of the immobilised 18/18 gel and a triazine-substituted gel as a control were equilibrated with water at 23–30°C at a flow-rate of 0.5 ml/min. Solutions (1 ml) of *N*-acetylglucosamine (5 m*M*), α-L-fucose (5 m*M*), α-D-glucose (10 m*M*), α-D-galactose (5 m*M*), α-D-mannose (5 m*M*), mannobiose (5 m*M*), mannan (1 mg/ml) and sialic acid (2 m*M*) were applied and the elution profiles in water recorded refractometrically. A qualitative index of interaction with the immobilised 18/18 ligand was obtained from the difference between the retention times for each saccharide on the affinity and control columns.

2.7. Partition equilibrium experiments

Interactions between glucose oxidase and *p*-nitrophenylglycosides with immobilised 18/18 in the Tris-chloride buffer (pH 7.0) were characterised quantitatively with a simple partition equilibrium protocol [19]. Reaction mixtures were prepared by adding either glucose oxidase (1 mg/ml) or *p*-nitrophenyl glycoside solutions (1 ml) to aliquots of affinity gel (20 μmol 18/18 ligand/g moist weight gel; 0.1 g moist weight) in a series of test tubes, which were sealed with parafilm and allowed to equilibrate for 24 h at 18–20°C. The concentration of glucose oxidase in each liquid phase, [A], was measured spectrophotometrically at 280 nm, the wavelength being adjusted to 305 nm for determination of the corresponding concentration in experiments with *p*-nitrophenyl glycosides as partitioning solute [20,21]. Total concentrations of the various partitioning solutes, $[\bar{A}]$, were based on the distribution of the added amount in a volume of 1.08 ml, the volume of liquid phase deduced from experiments with potassium chromate as partitioning solute.

Results for the dependence of the concentration of matrix-bound solute, $([\bar{A}] - [A])$, upon its free concentration, $[A]$, were analysed in terms of the rectangular hyperbolic relationship:

$$([\bar{A}] - [A]) = K_{AX}[A][\bar{X}]/(1 + K_{AX}[A]) \quad (1)$$

where K_{AX}, the effective association equilibrium constant, and $[\bar{X}]$, the corresponding total concentration of matrix sites, are the two curve-fitting parameters to emanate from the analysis. For display purposes, the Scatchard linear transform of Eq. (1) [21]:

$$([\bar{A}] - [A])/[A] = K_{AX}[\bar{X}] - K_{AX}([\bar{A}] - [A]) \quad (2)$$

has been used to facilitate recognition of the extent to which results conform with rectangular hyperbolic description.

2.8. Competitive binding studies

An additional series of partition experiments was designed to evaluate the equilibrium constants (K_{SX}) for the binding of methyl-mannoside and *p*-nitrophenyl-mannoside to the affinity matrix by virtue of their competitive effects on the partition of glucose oxidase. The same protocol was adopted for these experiments except that the enzyme solutions were prepared in the Tris-chloride buffer (pH 7.0) supplemented with either methyl-mannoside (1 m*M*) or *p*-nitrophenyl-mannoside (100 μ*M*).

Results from these experiments, which reflect only partial competition in the sense that the affinity matrix exhibits a greater capacity for monosaccharides than for glucose oxidase, have been analyzed in terms of the expression [22]:

$$(r_o - r)(1 + K_{AX}[A]) = K_{AX}[A]K_{SX}[S]/\{1 + K_{AX}[A] + K_{SX}[S]\} \quad (3)$$

The binding function for glucose oxidase, *r*, is obtained from experimental data reflecting the presence of competitor (*S*) by means of the relationship

$$r = ([\bar{A}] - [A])/[\bar{X}]_A \quad (4)$$

where $[\bar{X}]_A$ is the total concentration of matrix sites for glucose oxidase already determined: r_o, the corresponding value in the absence of competing ligand, was evaluated as:

$$r_o = K_{AX}[A]/(1 + K_{AX}[A]) \quad (5)$$

where K_{AX} is also the parameter deduced from analysis of partition experiments with enzyme alone. On the grounds of approximate constancy of [*S*], the free concentration of competing ligand, results of the competitive binding experiments were analyzed in terms of Eq. (3) to obtain the product of [*S*] and K_{SX}, the ligand-matrix binding constant, as the single parameter of unknown magnitude. Evaluation of K_{SX} was then based on the magnitude of this parameter and the value of [*S*] obtained from the expression:

$$[S] = [\bar{S}] - K_{SX}[\bar{X}]_S[S]/(1 + K_{SX}[S]) \quad (6)$$

which defines the free concentration of competing ligand in the absence of enzyme in terms of its total concentration ($[\bar{S}]$) and the effective total concentration of matrix sites for monosaccharidic solutes ($[\bar{X}]_S$).

2.9. Optimisation of elution conditions

Columns (0.9×6.2 cm) containing 1 ml of the ligand 18/18 (30 μmol/g moist weight gel) were pre-equilibrated with the buffer (10 m*M* Tris, 200 m*M* NaCl, 20% (v/v) ethylene glycol, 1 m*M* Mn^{2+} and 1 m*M* Ca^{2+} at pH 7.0). Glucose oxidase (1 mg/ml; 1 ml) was applied, the column washed with the same buffer and 2 ml fractions were collected at a flow-rate of 0.4 ml/min. Elution of bound GOx was effected with various 0.5 *M* sugar solutions: α-D-methyl mannoside, α-D-methyl glucoside, α-D-mannose, α-D-glucose and *N*-acetyl glucosamine. For another similar column pre-equilibrated with buffer devoid of the metal ions (Mn^{2+} and Ca^{2+}), the bound GOx was eluted using 0.5 *M* α-D-methyl mannoside.

2.10. Studies of the interaction between ligand 18/18 and α-D-methyl mannoside by ^{1}H-NMR spectroscopy

The ^{1}H-NMR spectra of α-D-methyl mannoside (10 mg, 50 μmol) and ligand 18/18 (19 mg, 50 μmol) in 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-methyl mannoside was performed to enable assignment of the peaks in this molecule. A few drops of water-d_2 was added to the solution of α-D-methyl mannoside and mixed thoroughly before the ^{1}H-NMR spectrum was retaken. To a fresh solution of α-D-methyl mannoside (10 mg, 50 μmol) in 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-methyl mannoside:ligand 18/18 mixture was also observed at 60°C and again at room temperature.

3. Results and discussion

3.1. Solid phase combinatorial synthesis and assessment of a second generation glycoprotein-binding ligand library

Lead optimisation of the known glycoprotein-binding ligand 8/10 was achieved by constructing a second generation solid-phase library that explored chemical space proximal to the hit ligand, followed by screening to select the refined lead and selecting a final ligand that is able to be readily synthesised at scale. The ligand library was assessed for its ability to bind and selectively elute the mannoprotein, glucose oxidase [12]. Fig. 1 shows the proportions of GOx bound and eluted with 0.5 *M* α-D-methyl-mannoside for 40 individual members of the solid-phase library. Inspection of the data shows that almost all ligands bearing the 5-aminoindan (18) substituent both quantitatively bound and eluted GOx, whilst ligands bearing the ethylenediamine (21) functionality, exhibited almost quantitative binding but virtually no elution under the conditions selected for screening. It is conceivable that ligands bearing the ethylenediamine substituent would behave as an anion exchanger towards the negatively charged GOx (p*I* 4.2). One ligand, the homobifunctional 18/18 (Fig. 2), displayed exceptional binding of GOx with almost 96% of the adsorbed glycoprotein eluted specifically with α-D-methyl-mannoside. A similar trend was observed when the ligand library was assessed for its ability to bind the mannoprotein ribonuclease B. Members of the library were relatively ineffectual at binding the aglycoprotein, ribonuclease A.

On the basis of these screening studies, the monofunctionality and the ease of synthesis and characterisation, the homobifunctional ligand 18/18 comprising 5-aminoindan bis-substituted on the triazine nucleus (Fig. 2), was selected for solution synthesis and further evaluation as a mannoprotein-binding ligand.

3.2. Solution synthesis of ligand 18/18 (2-(5-aminoindan)-6-(5-aminoindan)-4-chloro-s-triazine)

In order to prevent bis-substitution of cyanuric chloride when 5-aminoindan was used in excess and to generate a monosubstituted intermediate, a solution of 5-aminoindan was added dropwise to the slurry of cyanuric chloride. However, a slight excess of 5-aminoindan in the final mixture ensured the completion of the reaction within 5 min as observed by the disappearance of cyanuric chloride and the formation of 2-(5-aminoindan)-4,6-dichloro-*s*-triazine by TLC. The product was precipitated by adding water, and excess reactant, 5-aminoindan, was removed by washing the precipitate with copious amounts of water. The product was obtained in a yield of 98.4% and was confirmed essentially pure by ^{1}H-NMR. The compound had a distinct melting point at 137.8°C and an R_f of 0.364 on 0.25-mm silica gel using a solvent of hexane:ethyl acetate, 6:1 (v/v). The ^{1}H-NMR spectra of 5-aminoindan and 2-(5-aminoindan)-4,6-dichloro-*s*-triazine are shown in Fig. 3a and b respectively. The peaks on the spectra for these two compounds have been assigned. Downfield shifts in the aryl protons (Hb: 6.86–7.41, Hc: 6.52–7.54 and Ha: 6.42–7.21), with the Hc proton being affected the most, indicates the formation of a product that reacts through the 5-amino group (Fig. 3b). Furthermore, the disappearance of the amino peak on 5-aminoindan strongly suggests its substitution onto cyanuric chloride. Mass spectral analysis of the product, $(M+H)^+$:$C_{12}H_{10}N_4Cl_2$ showed a major fragment peak at 280.9 corresponding to the molecular weight of 2-(5-aminoindan)-4,6-dichloro-*s*-triazine.

The formation of 2-(5-aminoindan)-6-(5-aminoindan)-4-chloro-*s*-triazine was complete within 15 h at 23°C as observed by the disappearance of 2-(5-aminoindan)-4,6-dichloro-*s*-triazine by TLC. The product was precipitated in water, and excess reactant, 5-aminoindan, was removed by washing the precipitate with copious amounts of water. The product, a light brown powder, was obtained in a yield of 96.2% and was shown to be pure by ^{1}H-NMR. The compound had a distinct melting point at 175.8°C with an R_f of 0.227. The ^{1}H-NMR spectrum of the product (Fig. 3c) was very similar to the mono-substituted triazine, 2-(5-aminoindan)-4,6-dichloro-*s*-triazine; however, the Hb, initially seen as a doublet, was observed as a broad singlet. On raising the temperature of the compound to 35°C, the Hb sharpened-up to appear again as a doublet. This could be explained by the rotational movement of the

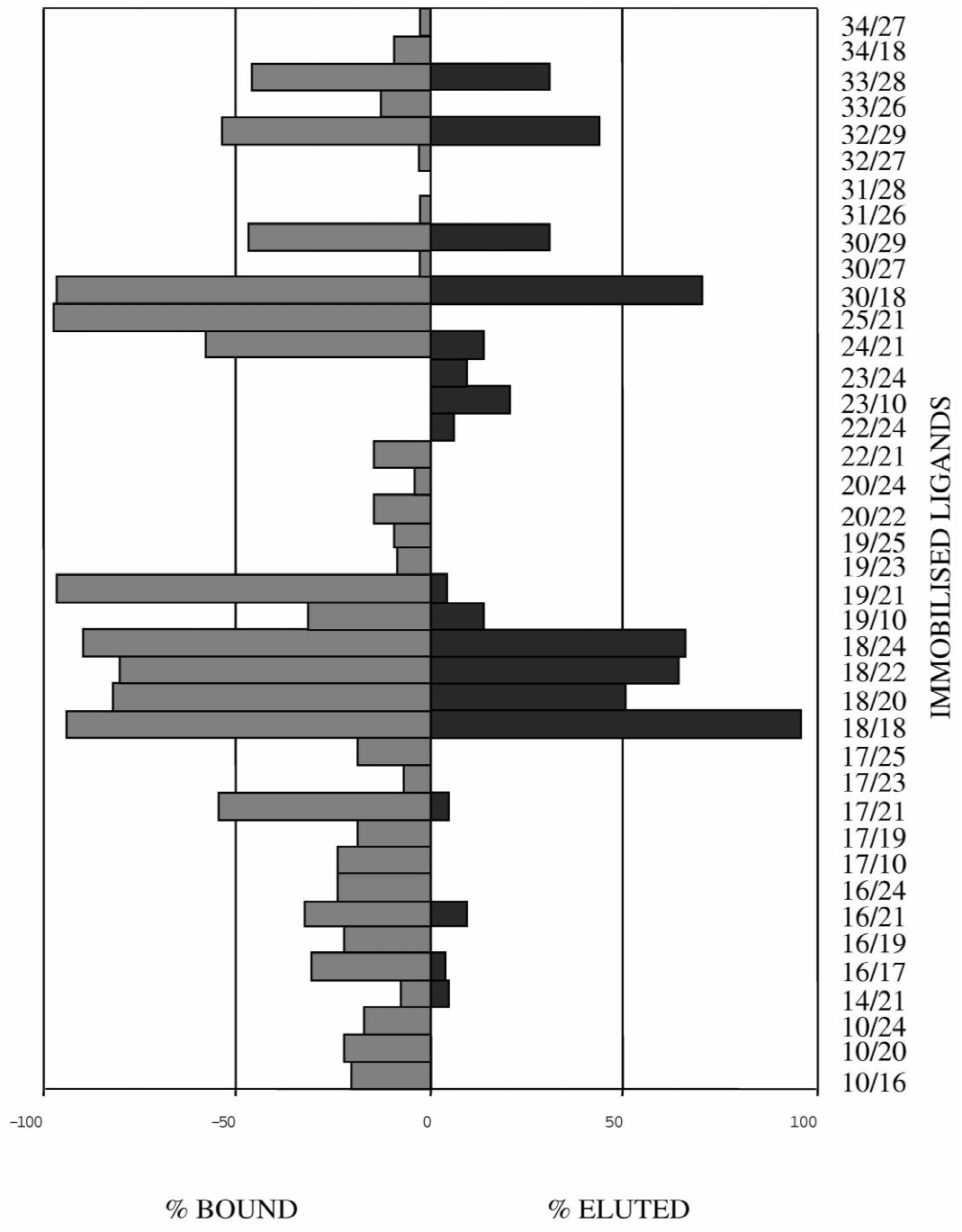

Fig. 1. Screening of the directed solid-phase ligand library for their ability to bind and elute *A. niger* glucose oxidase. The triazine-based ligands were synthesised on the solid support (25 μmol ligand/g moist weight gel), packed into columns (0.9×6.2 cm; 1 ml), and pre-equilibrated with 10 m*M* Tris–HCl buffer containing 0.2 *M* NaCl, 1 m*M* Ca^{2+} and 1 m*M* Mn^{2+} at pH 7.0. GOx (1 mg/ml; 1 ml) was applied, the column washed and the enzyme eluted with 0.5 *M* α-D-methyl-mannoside in the Tris buffer. The ordinate axis represents individual members of the combinatorial library coded according to the two substituents on the triazine scaffold. The numbers refer to the substituents listed in Section 2.3.

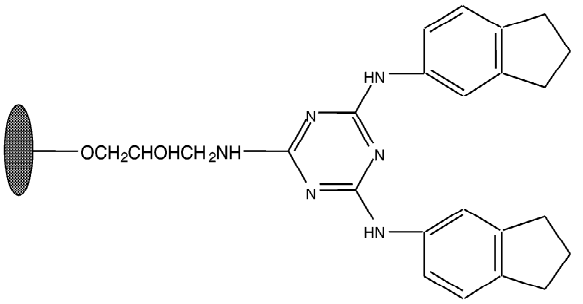

Fig. 2. The structure of the homobifunctional triazine affinity ligand 18/18.

two 5-aminoindan substituents on the triazine-scaffold at room temperature, thus appearing as a broad peak indicating additional conformations. When the temperature is raised, the movement of the 5-aminoindan molecules is so fast that they appear 'frozen' in one position and are observed as doublets. Mass spectral analysis of the product, $(M+H)^+$:$C_{21}H_{20}N_5Cl$ showed a major fragment peak at 378.1 corresponding to the molecular weight of 2-(5-aminoindan)-6-(5-aminoindan)-4-chloro-*s*-triazine.

3.3. Immobilisation of ligand 18/18 to agarose gels

The time of activation of the gel with epichlorohydrin significantly influenced the yield of epoxy-activated agarose. The epoxy concentration incorporated in the agarose increased linearly with time in the initial 145 min of activation, after which the incorporation rate was much slower until it

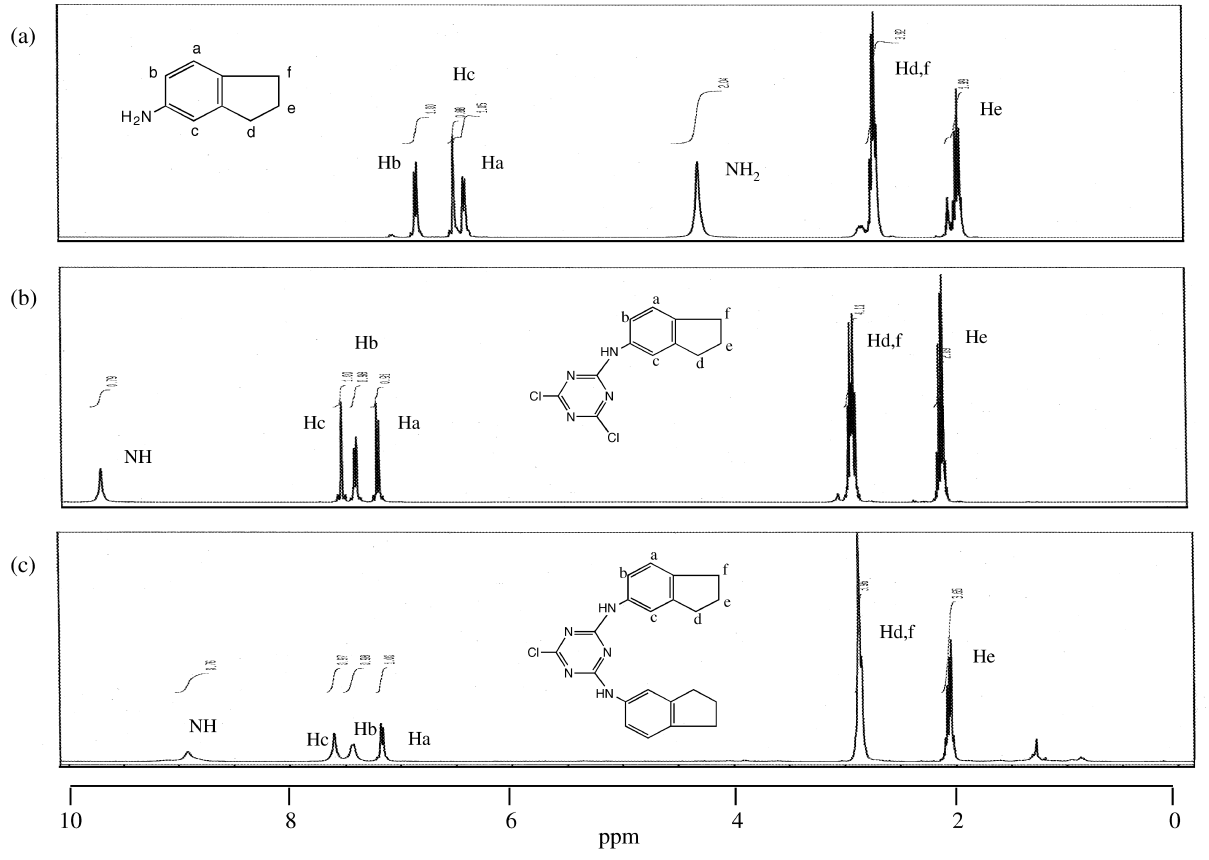

Fig. 3. ^{1}H-NMR Spectra of (a) 5-Aminoindan, (b) 2-(5-Aminoindan)-4,6-dichloro-*sym*-triazine and (c) 2-(5-Aminoindan)-6-(5-Aminoindan)-4-chloro-*sym*-triazine in acetone-d_6 at room temperature.

reached almost zero at 1080 min. A reasonable activation time for agarose with epichlorohydrin was 720 min, at which time approximately 34 μmol epoxy groups/g moist weight gel were incorporated.

Ligand 18/18 was immobilised on to the epoxy-agarose by amination and nucleophilic substitution. Immobilised 18/18 with ligand densities in the range 0–34 μmol/g moist weight gel was synthesised using the various epoxy-activated agaroses removed from the time course, and their effectiveness in binding GOx determined (Fig. 4). Immobilised 18/18 with ligand densities up to 11 μmol/g moist weight gel did not bind GOx, with almost all the enzyme appearing in the unbound fraction. Ligand densities between 18–22 μmol/g moist weight gel gave rise to an increasing percentage of GOx binding to the immobilised ligand with a retardation of the residual unbound fraction on the columns. Complete adsorption of GOx was observed at densities above 24 μmol/g moist weight gel. A similar trend was observed when 0.5, 1.0 and 2.0 mg/ml GOx was loaded onto the columns. This behaviour could be attributed to the fact that at ligand concentrations below 11 μmol/g moist weight gel, the 18/18 ligand molecules may be spaced too far apart to create an effective ligand-macromolecule complex which may require co-operativity for effective binding of the glycoprotein. It would appear that a ligand concentration of above 24 μmol/g moist weight gel of immobilised 18/18 is essential for complete adsorption of the glycoprotein, GOx. At this concentration the immobilised 18/18 ligands may be close enough to create an effective constellation for binding the glycoprotein. A similar effect was noted in the binding of human IgG to divinylsulphone (DVS) ligands [23], where the lack of binding at low ligand densities was explained by the requirement of IgG to bind to two or more sulphone ligands. In all further experiments, a ligand density of 30 μmol/g moist weight gel of 18/18 was used to achieve maximum adsorption of glycoproteins.

Immobilised 18/18 gels with polymethylene

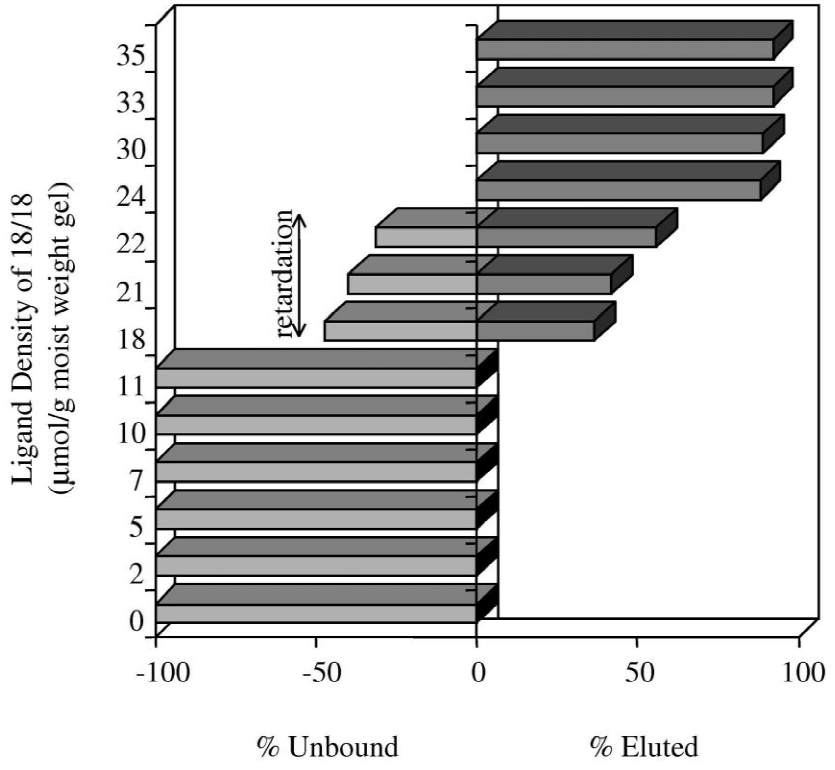

Fig. 4. The effect of immobilised ligand concentration on the ability of ligand 18/18 to bind glucose oxidase.

spacer arms of various lengths were synthesised and their effectiveness in binding GOx (1 mg/ml; 1 ml) determined. All adsorbents contained 30 μmol ligand/g moist weight gel and quantitatively bound the GOx loaded, although a decrease in the proportion of GOx eluted from 100 to 60% was observed as the number of methylene groups in the spacer molecule was increased from zero up to six. It would seem that the notionally longer spacer arms confer more hydrophobicity on the immobilised ligand and then contribute to tight binding of the glycoprotein. Furthermore, the longer polymethylene groups have a tendency to fold back on themselves in aqueous media; for example, the calculated distance of the 18/18 ligand from the matrix decreases from 10.30 to 8.45 Å when the spacer arm is increased from 5 to 6 methylene groups. It has been generally accepted that a bridge containing between 4 and 6 methylene groups must be interposed between the ligand and the lattice backbone in order to achieve optimal interaction with the target protein [24]. However, in the present work, maximum effectiveness for binding glucose oxidase was observed when the affinity ligand was attached directly to the aminated epoxy-activated gel and a nominal 5.71 Å away from the matrix backbone. This gel was used in all further experiments.

3.4. Chromatography of sugars on agarose-immobilised ligand 18/18

Preliminary testing of the efficacy of the 18/18-substituted agarose gel as an affinity matrix for saccharidic solutes entailed a comparison of the retention times obtained by chromatography of a range of sugars on an affinity column with those obtained on a control column of triazine-substituted agarose (Fig. 5). Despite the qualitative nature of these preliminary findings, an important feature to emerge is the demonstration that the 18/18-substituted agarose gel exhibits some selectivity in its affinity for saccharides. In that regard, the greater retardation of mannose-containing saccharides is pleasing in the sense that 18/18 was designed for maximal interaction with this monosaccharide [12]. An identical configuration of hydroxyl groups on C-2, C-3 and C-4 presumably accounts for the similarity of results for α-L-fucose and α-D-mannose. Further comparative aspects are examined later in quantitative studies of glycosides to avoid any complications arising from mutarotation at C-1.

3.5. Interaction of glucose oxidase with the 18/18 affinity gel

Since ligand 18/18 was developed to provide a stable affinity matrix for mannose-containing glycoproteins, a logical starting point for quantitative studies was the characterization of the interaction of the 18/18 affinity matrix with glucose oxidase, which has two mannose-rich glycosidic chains attached to each subunit of the dimeric enzyme [25].

Results of partition equilibrium studies of the interaction between glucose oxidase and ligand 18/18 were well described by Eq. (1) with respective values of $4.3(\pm 0.2)\times 10^5\ M^{-1}$ and $6.4(\pm 0.2)\ \mu M$ for K_{AX} and $[X]$ (data not shown). Of interest is the close conformity of the results with description in terms of this quantitative expression for a univalent partitioning solute, despite the existence of four glycosidic chains per enzyme molecule. Although such linearity is predicted [26] under circumstances where $([A]-[A])/[A] \ll 1$, that explanation seems untenable in the present instance. An alternative explanation is that spatial restrictions preclude multiple attachment of a glucose oxidase molecule to the affinity matrix [19]. Irrespective of which of these two situations prevails, the value of $4.3\times 10^5\ M^{-1}$ obtained for K_{AX} by means of Eq. (1) refers to the stoichiometric parameter for 1:1 complex formation, which is the product of glucose oxidase valence and the intrinsic binding constant for interaction of an enzyme site with the affinity matrix. On the basis of enzyme tetravalence, the intrinsic binding constant for the interaction of immobilized ligand 18/18 with an enzyme site is $1.1\times 10^5\ M^{-1}$. The other point of interest to emerge from the analysis is that the total concentration of matrix sites, $[X]=6.4\ \mu M$, equivalent to a matrix capacity of 68 nmol glucose oxidase per g moist-weight gel, and a value which contrasts markedly with the analytical ligand density of 20 μmol 18/18 ligand per g moist-weight gel.

Scatchard plots of results for the interactions of *p*-nitrophenyl derivatives of four monosaccharides are presented in Fig. 6. Several features are to be

noted: (i) Results for the D-mannoside, L-fucoside and D-glucoside are essentially superimposable, and have therefore been analysed according to Eq. (1) as a combined set to obtain values of $1.9(\pm 0.3)\times 10^4$ M^{-1} and $105(\pm 8)$ μM for K_{AX} and $[X]$, respectively; (ii) the interaction of matrix sites with *p*-nitrophenylgalactoside is slightly weaker, a value of 1.2×10^4 M^{-1} for K_{AX} being signified by the broken line in Fig. 7, which is based on the mean of the experimental data and the $[X]$ of 105 μM for the other *p*-nitrophenylglycosides; (iii) the much greater value of $[X]$ for monosaccharides than for glucose oxidase almost certainly reflects to a large extent the fact that binding of enzyme to a single matrix site eliminates many additional potential sites because of shielding from access by other glucose oxidase molecules, the so-called parking problem [27]: there is likely to be a 30- to 40-fold difference in the length of the polymer chain shielded by attachment of an enzyme and a *p*-nitrophenylglycoside molecule; (iv) On the grounds that this capacity of 1.1 μmol/g gel for monosachcarides still represents only 6% of the analytical 18/18 composition, the creation of an affinity site may well require juxtaposition of several 18/18 residues in a specific configuration. Indeed, the sandwiching of a mannose residue between 18/18 residues is suggested by modelling studies of the interaction. Such possible involvement of several immobilized ligand molecules in an affinity site was also raised to account for the lack of GOx binding by gels with a low level of ligand substitution.

The fact that the *p*-nitrophenyl glycosides bind to immobilised 18/18 attached to agarose certainly points to involvement of the carbohydrate portion of glucose oxidase in its interaction with the affinity matrix. Further evidence for the action of the agarose-immobilized 18/18 as an affinity matrix for glycoproteins is obtained from competitive binding studies of the effects of two monosaccharides, the methyl and *p*-nitrophenyl glycosides of mannose, on the partition equilibrium for glucose oxidase.

3.6. Displacement of glucose oxidase by monosaccharides

The effects of 69 μM *p*-nitrophenyl-mannoside and 926 μM methyl-mannoside on the interaction of glucose oxidase with the 18/18 affinity matrix are summarised in Fig. 7 and demonstrate their essential equivalence. Although the data are not well described by the mandatory linear relationship [Eq. (3)], the nature of the ordinate parameter is such that a two-fold change in $(r_0 - r)(1 + K_{AX}[A])$ for the

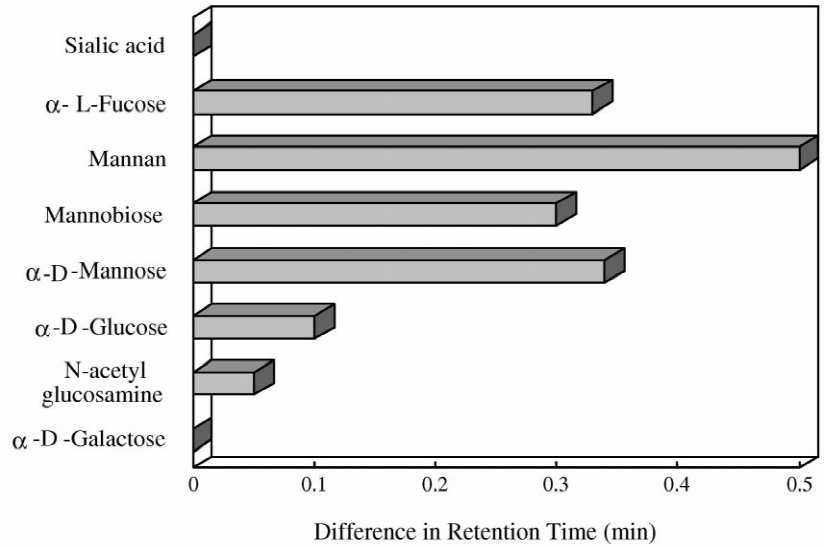

Fig. 5. Retardation of various sugars in chromatography on agarose-immobilised 18/18, the results being presented as the difference between retention times on columns (0.9×1.6 cm) of affinity gel and triazine-substituted agarose.

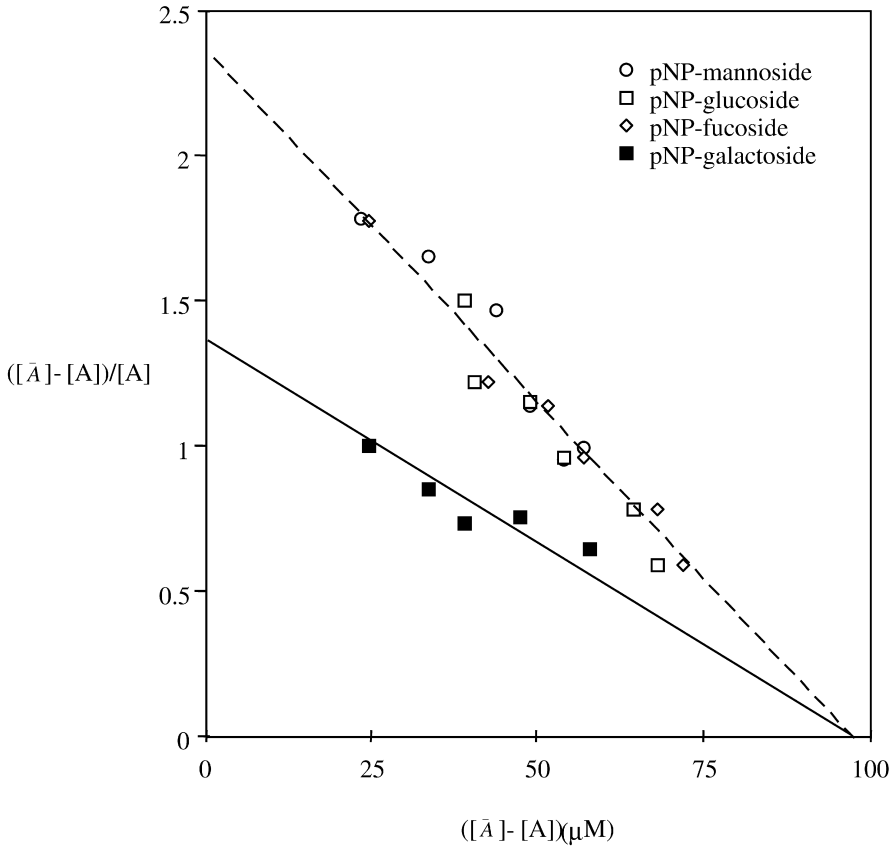

Fig. 6. Scatchard plots for the interactions of *p*-nitrophenyl glycosides of D-mannose (♦), L-fucose (s), D-glucose (■) and D-galactose (✖) with agarose-immobilised 18/18; ———, best-fit description of the combined $\{([\bar{A}] - [A])/[A]\}$ data set for the first three glycosides; - - -, line joining the mean of the galactoside data to the abscissa of the other plot ($[\bar{X}]$).

higher glucose oxidase concentrations would result from an uncertainty of only 0.03 in an *r* value of 0.6. We therefore proceed with analysis in terms of Eq. (3). Analysis of each (*r*, [*A*]) combination in terms of Eq. (3) yields a mean value of 0.56 for the product $K_{SX}[S]$ for either competitor, the standard deviation (±0.14) also being the same for both glycosides. That description and its envelope of uncertainty (±2 SD) are shown as the solid and broken lines respectively in Fig. 7. It now remains to estimate values of the free glycoside concentrations in order to delineate magnitudes of K_{SX} for the two saccharides.

On the basis of Eq. (6) with $[\bar{X}]_S = 105$ μ*M* (Fig. 6), the free concentration of *p*-nitrophenylmannoside in the absence of enzyme is calculated to be 31 μ*M*, a value that increases to 32 μ*M* in the experiment with highest enzyme concentration as the result of an effective decrease in $[\bar{X}]_S$ from 105 to 102 μ*M* due to the binding of 3 μ*M* A. Combination of the estimate of 0.56 for the product $K_{SX}[S]$ with the mean value of 31.5 μ*M* for [*S*] yields a K_{SX} of $1.8(\pm 0.3)\times 10^4\ M^{-1}$, which agrees with the estimate of $1.9\times 10^4\ M^{-1}$ inferred from Fig. 6 for the same interaction. Corresponding treatment of the other system leads to a value of 890 μ*M* for [*S*] and a K_{SX} of $600(\pm 300)\ M^{-1}$ for a competitive methylmannoside–matrix interaction.

The analyses of results presented in Figs. 6 and 7 are, of course, based on an unsubstantiated premise that the accessible liquid-phase volume of 1.08 ml determined for potassium chromate also applies to

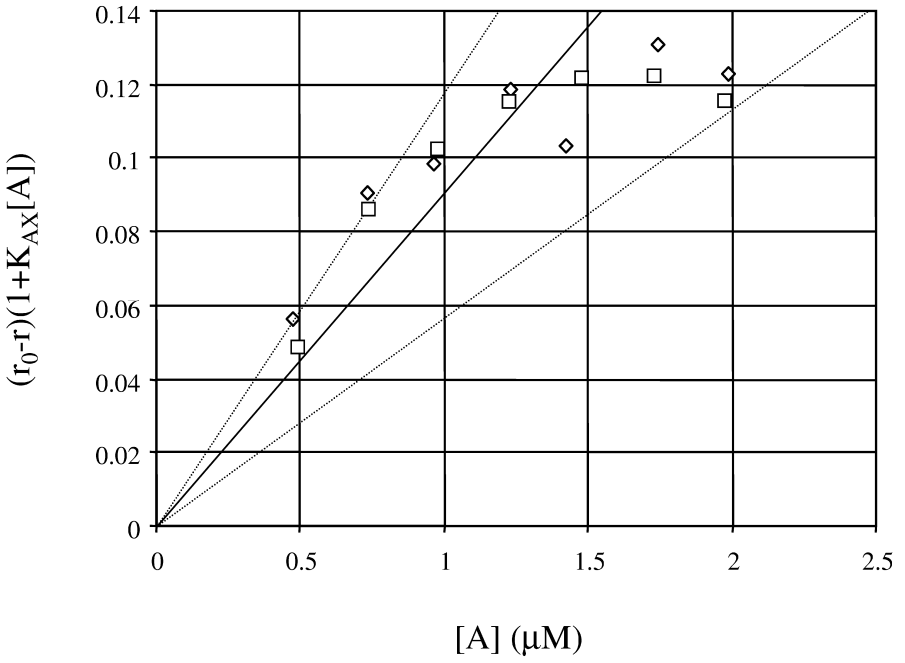

Fig. 7. Effect of the presence of 69 μ*M* *p*-nitrophenyl mannoside (♦) and 926 μ*M* methyl mannoside (*n*) on the interaction of glucose oxidase with agar-immobilized 18/18. ———, theoretical relationship for competitive inhibition based on the mean value of 0.56 obtained for $K_{SX}[S]$ by analysis of either data set in terms of Eq. (3): - - -, corresponding envelope of uncertainty (±2 SD).

glucose oxidase and the monosaccharidic glycosides — an approximation that is most suspect for the enzyme. Because of their subjectivity to the degree of accord with this approximation, the magnitudes of the various parameters cannot be regarded as accurate estimates. Fortunately, however, considerations of the findings to provide quantitative support for the involvement of carbohydrate-mediated binding in the adsorption of glucose oxidase to agar-immobilised 18/18 withstands acceptance of the binding parameters as approximate values. In other words, these partition equilibrium studies have sufficed to establish that the intended goal of developing 18/18-substituted agarose as a potential affinity matrix for glycoproteins has been achieved.

3.7. Optimisation of elution conditions

The elution of bound GOx from immobilised 18/18 using a variety of sugar solutions at 0.5 *M* concentration showed α-D-methyl mannoside (and mannose) followed by α-D-methyl glucoside (and glucose) to be effective in eluting the bound glycoprotein. Bound glycoproteins are commonly eluted with the sugar or an analogue for which the lectin has an affinity. For instance, mannose-rich glycoproteins binding to concanavalin A are often eluted with α-D-methyl mannoside while galactose-containing glycoproteins binding to soybean agglutinin are eluted with α-D-*N*-acetyl galactosamine [4,28,29]. The preference for mannoside over the other sugars by ligand 18/18 from this and earlier studies [12] confirms the selectivity of the ligand for mannose-type sugars. Exclusion of metal ions, Mn^{2+} and Ca^{2+}, in the equilibration buffer had no effect on the binding of GOx, indicating its non-requirement by the ligand for binding the glycoprotein — a situation that contrasts with that for plant lectins such as concanavalin A [30–32] and lentil lectin [33], for which Mn^{2+} and Ca^{2+} are required for effective binding of the glycoproteins.

3.8. Studies of the interaction between ligand 18/18 and α-D-methyl-mannoside by ^{1}H-NMR spectroscopy

The nature of the interaction between ligand 18/18 and α-D-methyl mannoside was studied using ^{1}H-NMR spectroscopy. The ^{1}H-NMR spectra of (a) ligand 18/18, (b) α-D-methyl mannoside and (c) the ligand 18/18:α-D-methyl mannoside complex is shown in Fig. 8. Proton peaks on α-D-methyl mannoside (Fig. 8b) were assigned after irradiating the individual peaks on the mannoside, while the hydroxyl clusters at 3.06–3.13 ppm (3 hydroxyls) and 2.70–2.73 ppm (CH_2OH) were identified on adding deuterated water (D_2O) 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-D-methyl mannoside complex (Fig. 8c) strongly suggests the formation of hydrogen bonds with the ligand 18/18. Furthermore, shifts in the H_1 (4.59 to 5.63 ppm) and H_2 and H_5, (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 of carbohydrate binding proteins [34–36].

To ensure that the disappearance of the hydroxyl groups is in fact due to the formation of hydrogen bonds, the ligand 18/18:α-D-methyl mannoside 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 the hydroxyl clusters, and on cooling, these hydroxyl groups were seen to disappear again, confirming the formation of hydrogen bonds between ligand 18/18 and α-D-methyl mannoside.

3.9. Molecular modelling

Molecular modelling was performed to support the

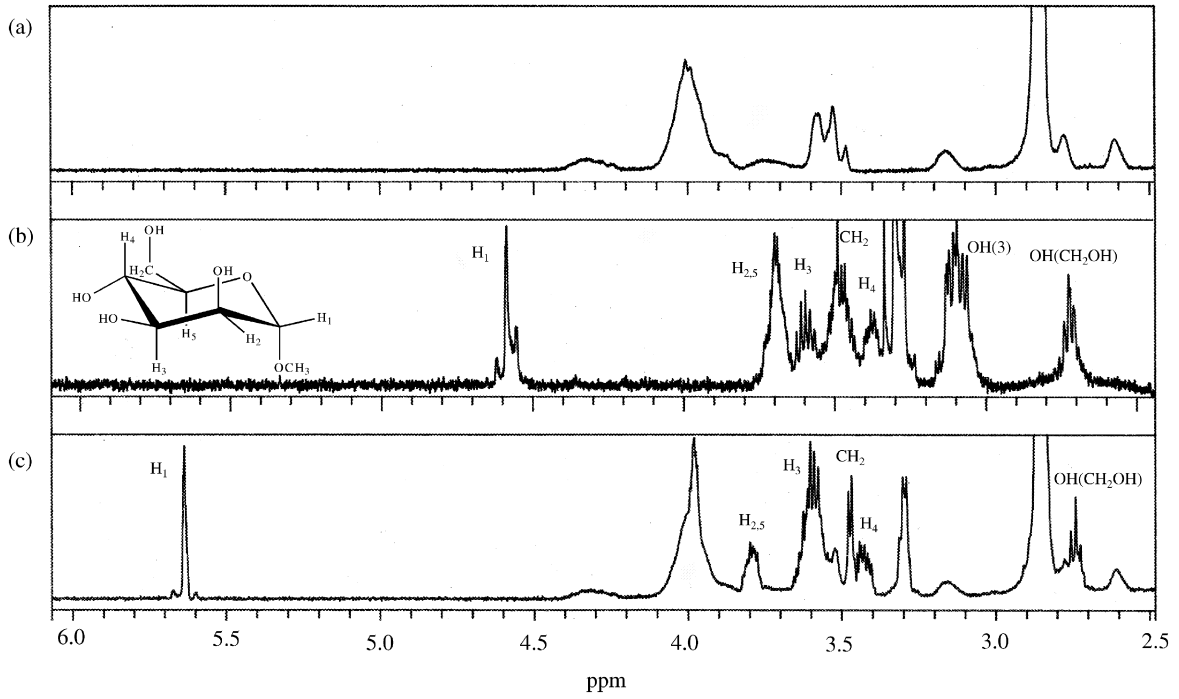

Fig. 8. ^{1}H-NMR Spectra of (a) ligand 18/18, (b) α-D-methyl-mannoside and (c) the ligand 18/18: α-D-methyl-mannoside complex in acetonitrile-d_3 at room temperature.

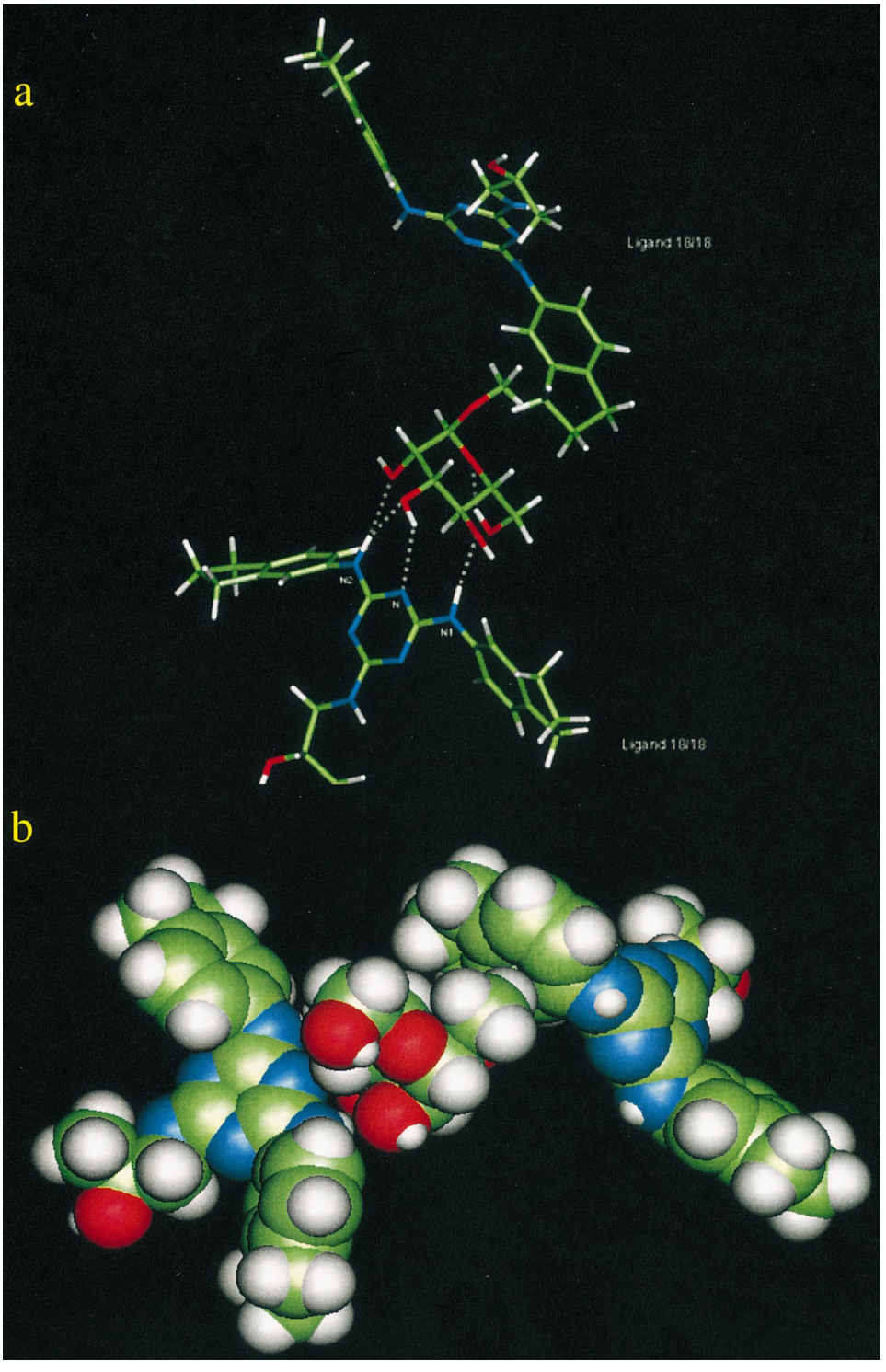

Fig. 9. A model showing putative hydrogen bonds and hydrophobic interaction between two molecules of 18/18 and α-D-methyl-mannoside: (a) A liquorice bond model, and (b) a van der Waal's model depicting the interaction between two molecules of 18/18 and α-D-methyl-mannoside.

above evidence for the nature of interaction between the ligand 18/18 and α-D-methyl mannoside. The structures of 18/18 and α-D-methyl mannoside were imported into the Quanta97 software and adjusted to their most likely orientation. Figs. 9a and b show the putative binding positions of ligand 18/18 and α-D-methyl mannoside. This conformation is considered practical since its energy constraint was zero and the energy of all the individual molecules is less than the thermal dynamic energy (~10 kcal/mol). The liquorice model (Fig. 9a) shows the formation of four hydrogen bonds between the hydroxyl groups at C-2, C-3 and C-4 of α-D-methyl mannoside and the bridging (N_1, N_2) and ring nitrogens (N) of a molecule of ligand 18/18.

A van der Waal's model of the same binding interaction is shown in Fig. 9b. The aromatic stacking of a second molecule of ligand 18/18 is 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 [34,37]. These models are supported by the experimental evidence for the requirement of the hydroxyl groups at C-2 and C-4, while the involvement of more than one molecule of ligand 18/18 has been implicated in both the ligand density and partition equilibrium studies. It is interesting to note that the triazine-scaffold plays a major role in the monosaccharide recognition. However, the fact that the triazine nucleus alone is a poor ligand suggests that both the 5-aminoindan (18) molecules and the triazine need to be present in a proper conformation for binding the saccharide.

4. Conclusions

Despite the simplicity of ligand 18/18, its specificity for saccharidic solutes bears a resemblance to that exhibited by concanavalin A, which also exhibits greater specificity for mannose and glucose than for galactose. For that system, X-ray crystallographic studies of the lectin-methylmannoside complex [38] signify involvement of the O on C-6 in hydrogen bonding as well as those on C-3 and C-4. Furthermore, the affinity for glucosides as well as mannosides may well relate to the fact that the hydrogen bond from the O on C-2 of mannose is via a water bridge to an adjacent subunit of the concanavalin A [38]. In similar vein, the comparable affinity of the synthetic ligand for glucose and mannose could well reflect interaction of the incorrectly configured C-2 hydroxyl group on glucose with the 18/18 residue on the other side of the complexed saccharide molecule. An apparent lack of involvement of the O on C-6 in the interaction of immobilised 18/18 with saccharides is implicated by the identity of results for *p*-nitrophenyl-L-fucoside and *p*-nitrophenyl-D-mannoside, and also by an affinity for methylmannoside, which is two orders of magnitude smaller than that exhibited by concanavalin A.

This work has clearly demonstrated the power of rational design and combinatorial chemistry for the development of a new affinity ligand for the potential purification of mannose-containing glycoproteins. Ligand 18/18 offers great promise as a potential affinity adsorbent for the purification of glycoproteins on a commercial scale — a situation where its advantage of greater stability to extreme conditions such as autoclaving is likely to outweigh those of greater affinity and specificity afforded by immobilised lectins.

5. Nomenclature

GOx glucose oxidase

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