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Galactosyl-biomimetic dye-ligands for the purification of *Dactylium dendroides* galactose oxidase

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Abstract

Two anthraquinone galactosyl-biomimetic dye-ligands comprising, as terminal biomimetic moiety, galactose analogues (1-amino-1-deoxy-β-D-galactose and D(+)-galactosamine) were designed for the enzyme galactose oxidase (GAO), using molecular modelling, synthesized and characterized. The biomimetic ligands were immobilized on agarose beads and the affinity adsorbents, together with a non-biomimetic adsorbent bearing Cibacron Blue 3GA, were studied for their ability to purify GAO from *Dactylium dendroides*. Both biomimetic adsorbents showed higher purifying ability for GAO compared to the non-biomimetic adsorbent, thus demonstrating their superior effectiveness as affinity chromatography materials. In particular, the affinity adsorbent comprising, as terminal biomimetic moiety, 1-amino-1-deoxy-β-D-galactose (BM1) exhibited the highest purifying ability for GAO. This affinity adsorbent did not bind galactose dehydrogenase, glucose dehydrogenase, alcohol dehydrogenase, or glucose oxidase. The dissociation constant (K_D) of the immobilized BM1 ligand with GAO was found to be equal to 45.8 μ*M*, whereas the binding capacity was equal to 709 U per ml adsorbent. Therefore, the BM1 adsorbent was integrated in a facile two-step purification procedure for GAO. The purified enzyme showed a specific activity equal to 2038 U/mg, the highest reported so far, ~74% overall recovery and a single band after sodium dodecylsulfate-polyacrylamide gel electrophoresis analysis. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Of all the separation techniques currently used in macromolecular separation science, affinity chromatography is considered to be the most specific, since it is based on the unique specificity, inherent in a ligand–biomacromolecule interaction, leading to the formation of stable and reversible complexes [1–4]. The efficiency of an affinity system is determined by factors such as specificity, capacity and stability, and these three are more or less dependent upon the nature of the affinity ligand [3–5].

Pseudobiospecific affinity chromatography, based on reactive commercial dye-ligands [6,7], has been

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extremely popular in the past and several of these dye adsorbents are now used in protein purification (for recent reviews see Refs. [8,9]). Among them, by far the most utilized is the triazine dye Cibacron Blue 3GA (CB3GA) which has been shown to interact with various enzymes and proteins. Affinity chromatography adsorbents based on reactive commercial dyes are of low cost, exhibit high binding capacity and are very stable and easy to regenerate [7]. The main problem with dyes is their relative lack of selectivity compared to biological ligands. In order to overcome this limitation, several rational attempts have been made with the aim of altering the binding specificity of a dye and, also, increasing its affinity for target proteins [8]. These attempts are based on the *biomimetic dye* concept [8,10,11], according to which new dyes that mimic the structure and binding of natural biological ligands of the targeted proteins are designed. For example, this can simply be realized after substitution of the terminal aminobenzene sulfonate moiety of Cibacron Blue 3GA with a suitable substrate-mimetic moiety [10,12–14]. Some of these ligands may be considered as bifunctional for they are recognized by several dehydrogenases as chimeric pseudo-substrates, with the anthraquinone moiety mimicking the natural coenzyme and the biomimetic one acting as a substrate-mimetic analogue [12,15]. The biomimetic dyes satisfy the need for increased purifying ability and specificity, and provide useful tools for designing simple and effective enzyme purification protocols. This approach has been successfully used in the case of (keto)carboxyl group-recognising enzymes, such as formate dehydrogenase [13], L-lactate dehydrogenase [12], and L-malate dehydrogenase [16], as well as glutathione-recognising enzymes [14].

New biomimetic dye-ligands can be designed either empirically, by substituting large number of substrate-mimetic structures to the parent dye [13,17], or rationally [12,14,15]. In the latter approach, molecular modelling techniques and the three-dimensional (3D) structure of the target enzyme, or, at least, the amino acid sequence of the target protein and the three-dimensional structure of a highly homologous protein, are employed to predict the optimum biomimetic ligand structure [12,14,15,18,19].

The biomimetic dye concept with respect to saccharo-biomimetic structures is examined, for the first time, in the present study. In particular, the design, synthesis and study of galactosyl-biomimetic ligands as well as the respective affinity adsorbents, suitable for the purification of the galactose-recognizing enzyme galactose oxidase (GAO), are presented. These immobilized biomimetic dyes are easy to synthesize analogues of the parent dichlorotriazine dye Vilmafix Blue A-R (VBAR), bearing galactosyl-like structures as biomimetic moiety. The purifying ability of the new galactosyl-biomimetic adsorbents was studied for galactose oxidase from *Dactylium dendroides*, in that a facile purification procedure for this enzyme was developed. The study on GAO purification is justified by the analytical and biotechnological potential of the enzyme and the lack of a rapid and efficient purification method. GAO is used as an analytical tool for the quantitative determination of galactose in blood and other biological fluids [20], as well as in glycoprotein engineering [21].

2. Experimental

2.1. Materials

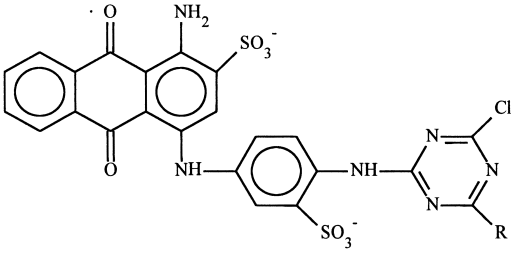
Galactose oxidase crude lyophilized powder (EC 1.1.3.9, from *D. dendroides*), glucose oxidase crude lyophilized powder (EC 1.1.3.4, from *Aspergillus niger*), 1-amino-1-deoxy-β-D-galactose (β-D-galactosylamine), D(+)-galactosamine (2-amino-2-deoxy-D-galactopyranose; chondrosamine), D(+)-galactose (minimum 99%), D(+)-glucose, *o*-toluidine, *o*-dianisidine, CB3GA, lipophilic Sephadex LH-20, Sepharose CL-6B, CM-Sepharose CL-6B and DEAE-Sepharose CL-6B were obtained from Sigma (St. Louis, MO, USA). β-Galactose dehydrogenase (EC 1.1.1.48, from *Pseudomonas fluorescens*), peroxidase (from horseradish, grade I), NAD^+ (crystallized lithium salt ~100%) and crystalline bovine serum albumin (fraction V) were obtained from Boehringer Mannheim (Germany). Nutrient broth (for microbiology) was obtained from Merck (Germany). F324 *P. fluorescens* biovar V1 was kindly donated by Professor G.J. Nychas (Laboratory of Microbiology and Biotechnology of Foods, Agricul-

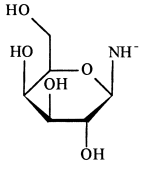
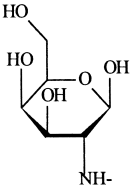
tural University of Athens). Baker's yeast and green peas were purchased from the local market. Glucose dehydrogenase was extracted from *P. fluorescens* and baker's yeast, while alcohol dehydrogenase was extracted from baker's yeast and green peas.

2.2. Synthesis and purification of the galactosyl-biomimetic dye-ligands

Biomimetic dye-ligands (Table 1, structures BM1, BM2) were synthesized by nucleophilic substitution at the dichlorotriazine ring of the parent dye (VBAR) by the amino-group of the galactose analogues (1-amino-1-deoxy-β-D-galactose and D(+)-galactosamine, structures BM1, BM2), as follows. Solid commercial VBAR (250 mg, 0.225 mmol dichloroform, purity 61.3%, w/w) was added to water (10 ml, 25 °C), and the solution was slowly introduced, under stirring, over a period of 20 min, to a solution (10 ml) of the galactose analogue (1.125 mmol). The pH was adjusted and kept at 8.5 throughout the reaction. The progress of the reaction was followed by TLC using the solvent system: 1-butanol–acetone–pyridine–toluene–water (5:4:4:2:4, v/v). A silver nitrate ammonia solution was used as a spray reagent for detecting the galactose-analogues in the newly synthesized dyes [22,23]. After the reaction was completed (at least 2 h, 25 °C, as judged by

Table 1
Characteristics of galactosyl-biomimetic dyes (BM1, BM2) for GAO and Cibacron Blue 3GA (CB3GA)



Dye-ligand (-R)	M_r (sodium salt)	mϵ (mM^{-1} cm^{-1}) in water	λ_{max} (nm) in water
BM1 	824.1	12.6	608
BM2 	824.1	11.7	610
CB3GA -*o*-HN*Benz*SO_3^-	839.5	12.6	622

TLC) the mixture was frozen at −20 °C and lyophilized. The lyophilized powder was stored desiccated at 4 °C.

Dye purification to homogeneity was achieved in two stages. During the preliminary stage inorganic and certain organic contaminants [24] were removed, while in the second stage complete purification of dyes was achieved by liquid column chromatography [25]. Preliminary purification was effected by dissolving crude dye (250 mg) in deionized water (20 ml) and stirring for 5 min at room temperature. The solution was extracted twice with diethyl ether (2× 25 ml), the aqueous phase concentrated (~3-fold) on a rotary evaporator and the dye precipitated by addition of cold acetone (50 ml). The precipitate was filtered (Whatman paper filter) and dried under reduced pressure. Pretreated crude dye (100 mg) from the first stage was dissolved in water–methanol (5 ml, 50:50, v/v) and filtered through a Millipore cellulose membrane filter (0.45-μm pore size). The dye solution was applied and chromatographed on a lipophilic Sephadex LH-20 column (30×2.5 cm) [25]. The purification of Cibacron Blue 3GA was performed according to a published procedure [25].

2.3. Spectroscopic characterization of dye-ligands

The absorption maxima (λ_{max}) of the purified dyes were determined from the absorption spectra of aqueous dye solutions (50 μ*M*) taken in the range 850–450 nm. The molar absorption coefficients (ϵ values) were calculated from the linear section of reference curves derived by plotting dye concentration versus absorption (620 nm, 20–100 μ*M*) [25].

2.4. NMR and MS analyses

NMR spectra were recorded on a Bruker AM 250 or 500 MHz spectrometer using standard pulse sequences. Samples were analyzed as solutions in [$^2H_6$]dimethyl sulfoxide (DMSO-d_6) or ^2H_2O.

Electron impact (EI) and fast atom bombardment (FAB) mass spectra were recorded on a VG ZAB/SE double focussing low/high resolution spectrometer. In the FAB spectra MNOBA and TGT matrices have been used. Electrospray ionization (ESI) spectra were run on a Finnigan LCQ DUO spectrometer.

2.5. Immobilization of dye-ligands

Immobilization of the dye-ligand was performed by modification of a published protocol [26]. To washed crosslinked agarose gel (Sepharose CL-6B, 1 g) was added a solution of purified dye in water (1 ml, the amount of dye is shown in Table 2), followed by NaCl solution (22%, w/v; 0.2 ml). The suspension was tumbled for 30 min at room temperature prior to the addition of solid sodium carbonate (at a final concentration of 1%, w/v). The reaction continued under shaking at 40 °C for the galactosyl-biomimetic dyes or at 60 °C for CB3GA. The time of immobilization reaction was varied, depending on the dye-ligand (Table 2). After completion of the reaction, dyed gels were washed sequentially with water (100 ml), 1 *M* NaCl (50 ml), 50% dimethyl sulfoxide (DMSO) (10 ml), 1 *M* NaCl (50 ml) and, finally, water (100 ml). The dyed gels were stored as moist gels in 20% methanol at 4 °C.

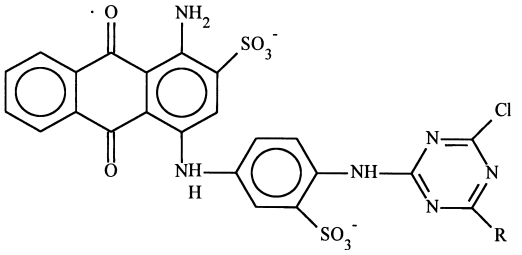
2.6. Determination of immobilized dye concentration

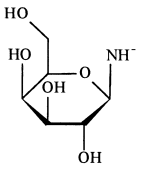
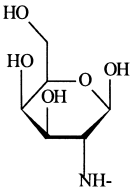
Determination of immobilized dye concentration was achieved according to Ref. [13]. The concentration of the immobilized dyes was calculated as micromoles of dye per gram moist mass gel, using the molar absorption coefficients shown in Table 2.

2.7. Assay of enzyme activity and protein

Galactose oxidase (GAO), galactose dehydrogenase (GalDH), glucose oxidase (GOD), glucose dehydrogenase (GDH) and alcohol dehydrogenase (ADH) assays were performed at 25 °C with the exception of GOD, which was performed at 35 °C. The assays were performed according to Refs. [27–31], respectively. All assays were performed in a double beam UV–visible spectrophotometer equipped with a thermostated cell holder (10-mm pathlength). For GAO, 1 U of enzyme activity is defined as the amount that produces a $\Delta A_{425\ nm}$ of 1.0 per min at the conditions of the assay. For the rest of the enzymes, 1 U of enzyme activity is defined as the amount that catalyses the conversion of 1 μmol of substrate to product per min. Protein concentration was determined by the method of Bradford [32] or by a

Table 2
Conditions and performance of immobilization reactions for purified dyes on agarose gel



Dye-ligand (-R)	mg dye/g moist gel (in reaction)	Time (h)	μmol dye/g moist gel (in adsorbent)	mϵ^{a} (mM^{-1} cm^{-1})
BM1 	10	18	2.4	10.4
BM2 	12	20	2.3	11.1
CB3GA -*o*-HN*Benz*SO_3^-	5	4.5	2.2	9.2

[a] Determined in medium identical to the one resulting after acid hydrolysis of the adsorbent [13]. Values were determined from 20-μ*M* dye solutions made in the above medium.

modified Bradford's method [33], using bovine serum albumin (fraction V) as standard.

2.8. Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed according to Laemmli [34] on a 0.75-mm-thick vertical slab gel (10×8 cm) containing 12.5 (w/v) polyacrylamide (running gel) and 2.5% (w/v) stacking gel, using a Hoefer SE 250 dual-slab cell unit. Protein samples, after dialysis against 25 m*M* Tris–HCl, pH 6.8, were mixed with an equal volume of treatment Tris–HCl buffer (125 m*M*, pH 6.8) containing SDS (4%, v/v), glycerol (20%, v/v), β-mercaptoethanol (β-MeSH; 10%, v/v), and bromophenol blue (0.002%, w/v). The samples were incubated at 75 °C for 5 min, applied to the wells, and run at a current of 20 mA per gel for 1 h. Protein bands were stained with Coomassie Blue R-250.

2.9. Cell cultures

P. fluorescens, strain 53, precultures were prepared by inoculating the bacterium into nutrient broth (Merck) (8.0 g in 1 l of deionized water, pH

7.0±0.2, 25 °C). Stock cultures were maintained in agar-nutrient broth dishes, stored at 4 °C. Cultures (200 ml) were prepared in erlenmeyer flasks, which were incubated on a rotary shaker (120 rpm) for 48 h at 25 °C. The cells were harvested by centrifugation at 4 °C (10 000 *g* for 25 min), washed three times with potassium phosphate buffer (50 m*M*, pH 7.6) and recentrifuged. This procedure yielded ~4 g cell paste/l culture. The cell paste was then frozen, lyophilized, and the dry lyophilized mass stored at −20 °C. Typically, this procedure yielded 1–1.5 g dry cell mass/l culture.

2.10. Preparation of cell extracts

Commercial lyophilized crude powder (10 mg) of *D. dendroides* was suspended in potassium phosphate buffer (100 m*M*, pH 7.0, 2 ml) and the suspension was centrifuged (5000 *g* for 20 min, 4 °C). The supernatant was dialyzed overnight at 4 °C against 2 l of 100 m*M* potassium phosphate buffer, pH 7.0. The dialysate was clarified through a Millipore cellulose membrane filter (0.45-μm pore size), affording specific activity, typically, 51.4 U GAO/mg (18.3 U GAO/ml extract, 3.7 U GAO/mg cell lyophilized powder).

P. fluorescens dry cells (1.5 mg for GalDH, 60 mg for GDH) were suspended in potassium phosphate buffer (100 m*M*, pH 7.0, 1 ml for GalDH, 1.5 ml for GDH) and disintegrated under sonication (Vibra Cell, 400 W, Sonics and Materials) (amplitude: 40%, 2-s sonication—5-s pause, eight cycles, 4 °C). Cell debris was removed by centrifugation (5000 *g* for 20 min, 4 °C) and the supernatant was dialyzed overnight at 4 °C against 2 l of 100 m*M* potassium phosphate buffer, pH 7.0. The dialysate was clarified through a Millipore cellulose membrane filter (0.45-μm pore size), affording, typically, 0.08 U GDH/ml extract (0.053 U GDH/mg dry cells). In the case of GalDH, before dialysis, the supernatant was enriched with commercial enzyme (*P. fluorescens* gene, expressed in *Escherichia coli*, ~0.8 U added/ml extract), in order to achieve a final specific activity of ~1.15 U GalDH/mg.

Commercial lyophilized crude powder (11 mg) of *A. niger* was suspended in potassium phosphate buffer (100 m*M*, pH 7.0, 1.5 ml). The suspension was centrifuged (5000 *g* for 20 min, 4 °C) and the supernatant was dialyzed overnight at 4 °C against 2 l of 100 m*M* potassium phosphate buffer, pH 7.0. The dialysate was clarified through a Millipore cellulose membrane filter (0.45-μm pore size), affording, typically, 11.2 U GOD/ml extract (1.5 U GOD/mg solid).

Commercial baker's yeast cells (1.3 g paste for GDH, 1.2 g paste for ADH) were suspended in potassium phosphate buffer (100 m*M*, pH 7.0, 1.5 ml) and disintegrated under sonication (amplitude 40%, 5-s sonication—5-s pause, 12 cycles, 4 °C). Cell debris was removed by centrifugation (14 000 *g* for 50 min, 4 °C) and the supernatant was dialyzed overnight at 4 °C against 2 l of 100 m*M* potassium phosphate buffer, pH 7.0. The dialysate was clarified through a Millipore cellulose membrane filter (0.45-μm pore size), affording, typically, an activity of 0.39 U GDH/ml extract (0.52 U GDH/g cell paste) and 5.5 U ADH/ml extract (7.3 U ADH/g cell paste).

Green peas (3.5 g) were suspended in potassium phosphate buffer (100 m*M*, pH 7.0, 15 ml), pulped using a pestle and mortar, and homogenized (Virtishear mechanical homogenizer, 10 000 rpm, 1 min, 4 °C). The homogenized suspension was filtered using cheese cloth and the filtrate was centrifuged (18 000 *g* for 40 min, 4 °C). The supernatant was dialyzed overnight at 4 °C against 5 l of 100 m*M* potassium phosphate buffer, pH 7.0. The dialysate was clarified through a Millipore cellulose membrane filter (0.45-μm pore size), affording, typically, an activity of 0.3 U ADH/ml extract (0.46 U ADH/g).

2.11. Affinity chromatography evaluation of dye adsorbents with GAO from D. dendroides extract

All procedures were performed at 4 °C. Galactose oxidase binding was assessed using analytical columns, each packed with 0.5 ml of each of the three dye adsorbents (2.3–2.4 μmol dye/g moist gel). Columns were equilibrated with 100 m*M* potassium phosphate buffer, pH 7.0. Dialyzed *D. dendroides* extract (1 ml, 19.9 U GAO, 0.39 mg protein) was applied to each analytical column. Non-adsorbed protein was washed off with equilibration buffer (4 ml). Bound GAO was eluted by 5 m*M* galactose in the equilibration buffer (6 ml). Collected fractions (2 ml) were assayed for GAO activity and protein [33].

The fractions with GAO activity were pooled and the specific activity was determined.

2.12. Ion-exchange chromatography of GAO from D. dendroides extract

All procedures were performed at 4 °C. Analytical columns were used, each packed with 0.5 ml CM-Sepharose CL-6B or DEAE-Sepharose CL-6B. Columns were equilibrated with 10 m*M* potassium phosphate buffer, pH 6.0, 6.5, 7.0, 7.5 and 8.0. Dialyzed *D. dendroides* extract (1.15 ml, 22.5 U GAO, 0.44 mg protein) was applied to each analytical column. Non-adsorbed protein was washed off with equilibration buffer (3 ml). The DEAE anion exchanger did not bind much of the enzyme, so activity and purification measurements were carried out on the throughput fraction. The CM cation exchanger bound the enzyme, therefore it was further washed with 2 ml of equilibration buffer containing 80 m*M* (pH 6.0, 6.5), 60 m*M* (pH 7.0) or 40 m*M* (pH 7.5) KCl. Bound GAO was eluted by 120 m*M* (7 ml, pH 6.0, 6.5), 100 m*M* (6 ml, pH 7.0) or 80 m*M* (8 ml, pH 7.5) KCl. Collected fractions (1 ml) were assayed for GAO activity and protein [33]. The fractions with GAO activity were pooled and the specific activity was determined.

2.13. Purification of GAO from D. dendroides extract

All procedures were performed at 4 °C.

2.13.1. Step 1: ion-exchange chromatography on CM-Sepharose CL-6B

Dialyzed extract (1.15 ml, 21.6 U GAO, 0.42 mg protein) was applied to a column of CM-Sepharose CL-6B cation exchanger (0.5 ml) previously equilibrated with 10 m*M* potassium phosphate buffer, pH 6.5. Non-adsorbed protein was washed off with 3 ml equilibration buffer, followed by 2 ml of equilibration buffer containing 80 m*M* KCl. Elution of GAO activity was carried out with 7 ml of equilibration buffer containing 120 m*M* KCl. Collected fractions (1 ml) were assayed for GAO activity and protein [33]. The fractions with GAO activity were pooled (7 ml) and the specific activity was determined.

2.13.2. Step 2: affinity chromatography on biomimetic dye adsorbent BM1

GAO activity eluted from the ion exchanger was dialyzed overnight against 2 l of 100 m*M* potassium phosphate buffer, pH 7.0. The dialysate (7 ml, 18.6 U, 0.075 mg protein) was applied to a column of biomimetic dye BM1-agarose (0.5 ml) which was previously equilibrated with 100 m*M* potassium phosphate buffer, pH 7.0. Non-adsorbed protein was washed off with 4 ml equilibration buffer. Bound GAO was eluted in equilibration buffer containing 5 m*M* galactose (6 ml). Collected fractions (2 ml) were assayed for GAO activity and protein (A_{280}, except for fractions with galactose, where the protein was determined by a modified Bradford's method [33]). The fractions with GAO activity were pooled (6 ml) and the specific activity was determined.

2.14. Determination of dissociation constant (K_D) and GAO-binding capacity of immobilized BM1 ligand

Determination of the K_D value of immobilized BM1 was performed essentially according to the method of Kasai and co-workers [35,36]. The column contained 100 mg wet mass (0.16 ml wet volume gel) of affinity adsorbent equilibrated with 100 m*M* potassium phosphate buffer, pH 7.0. A dilute solution of GAO (40.5 U GAO/ml, M_r for GAO 68 000) made in the above buffer, was continuously applied at a flow rate of 1.7 cm/min until the effluents had reached a steady maximum GAO activity. The fractions collected (typically 0.5 ml) were assayed for GAO activity. The mathematical calculations are described in Results and discussion.

Adsorbent capacity was calculated from the plot of eluting GAO activity versus effluent volume, and it is defined as the GAO units present in extract volume which is equal to effluent volume corresponding to 10% of the maximum eluting activity from 0.16 ml of adsorbent.

2.14.1. Docking of the galactosyl-biomimetic ligands into the galactose oxidase structure

The 1.7A structure of GAO (code 1GOG; [37]) was retrieved from the PDB [38]. The biomimetic ligands were constructed with the aid of the HICUP database of heterocompounds [39]. The CBD, GLA

and GLB entries, containing Cibacron Blue, α-D-galactose and β-D-galactose, respectively, were edited and combined. The ligand was manually positioned in the active site of the model within the program O [40]. Molscript [41] was used to produce structural figures.

3. Results and discussion

3.1. Design of galactosyl-biomimetic ligands (BMs) for galactose oxidase (GAO)

Two anthraquinone galactosyl-biomimetic dye-ligands, analogues of the parent anthraquinone dichlorotriazinyl dye VBAR, were designed to mimic galactose, the natural substrate of GAO. Each biomimetic dye exhibits a terminal galactosyl-biomimetic moiety, which mimics the enzyme substrate, while the anthraquinone chromophore moiety, which usually acts as a nucleotide coenzyme pseudo-analogue [12,14,15], is not expected to have such a role with this particular enzyme. Instead, this chromophore moiety may act as a mixed function ''spacer'' which protrudes the galactosyl moiety to the solvent, thus facilitating the interaction with GAO.

Previous hypotheses about the mode of galactose binding to GAO [42] were used to guide the positioning of this portion of the biomimetic ligands. Modelling was first carried out with four biomimetic structures containing either α-D-galactose or β-D-galactose. As previously observed [42], a plausible binding mode for galactose can be obtained with the 6-OH interacting directly with the copper atom bound at the active site of GAO (Fig. 1). Additional

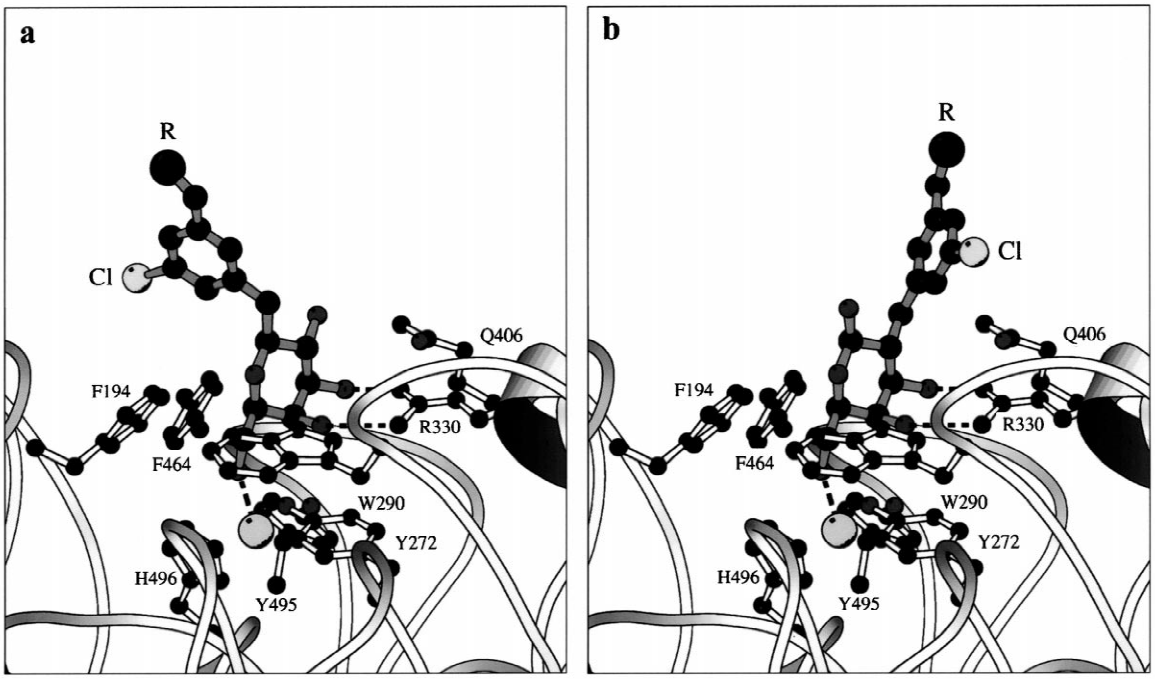


Fig. 1. Comparison of the predicted binding modes of (a) C1-linked and (b) C2-linked β-D-galactosyl-biomimetic ligands to the active site of GAO. Outside the galactose portion no specific binding is predicted so that only a representative conformation is illustrated. The protein is drawn schematically with only active site residues individually shown in a ball-and-stick representation and labeled. Biomimetic structures are shown larger and grey-shaded in order to help distinguish them from protein. The large isolated sphere is the copper atom. Dotted lines are used for possible hydrogen bonds and to illustrate the interaction of biomimetic O6 with the copper atom. The Cl atom, the site of ligation to the column matrix, and the undisplayed remainder anthraquinone diaminobenzosulfonate moiety of the ligand are labelled Cl and R, respectively.

hydrogen bonds may be made between the 3- and 4-OH groups of galactose and Arg330, and hydrophobic contacts between the galactose ring and phenylalanine residues 194 and 464 (Fig. 1). The binding mode can explain aspects of the substrate specificity of GAO [42]. Also in support of this binding mode, not previously noted, is the close correspondence between the predicted positions of the O6 and O4 atoms and two crystallographically observed water molecules; clearly these positions are chemically suitable for hydroxyl groups. In this configuration the galactose ring exposes both its 1- and 2-OH (the sites of attachment to the remainder of the ligands) to the solvent (Fig. 1).

Cibacron blue dye (CB3GA) is widely used for purification of NAD(P)(H)-dependent-enzymes since its largely hydrophobic anthraquinone moiety has an affinity for dinucleotide binding sites [43]. GAO does not use NAD(P)(H) and a search of the molecular surface near the active site failed to located significant hydrophobic patches. Presumably, therefore, the anthraquinone moiety does not bind significantly to GAO, instead remaining solvent-exposed (Fig. 1). The α or β anomericity of galactose on formation of the ring structure, affects the C1 asymmetric centre alone. For both immobilized anomeric biomimetic BM1 structures, solvent exposed anthraquinone conformations can be readily achieved (Fig. 1 for β-BM1; data are not shown for α-BM1). The different 1-OH positions seen for the anomeric BM2 structures are predicted to have negligible effects on affinity (Fig. 1 for β-BM2; data are not shown for α-BM2).

3.2. Synthesis of biomimetic dye-ligands and adsorbents

The two biomimetic dyes and the commercial dye CB3GA were purified to homogeneity in two stages, prior to their characterization and study. Following a preliminary treatment, complete dye purification was achieved by liquid column chromatography on Sephadex LH-20, as confirmed by TLC analysis (single blue bands).

The starting commercial dye preparations are known to contain added buffers, stabilizers, and fillers, on top of organic reactants [44,45]. The preliminary stage was introduced to remove unreacted cyanuric acid and organic-phase soluble contaminants [45], while further purification of the dye during the second stage was effected by liquid column chromatography. Column chromatography on Sephadex LH-20, which was included in order to remove unwanted salts and dye impurities, has proved to be a rather effective technique for purifying all dyes engaged here. Table 1 summarises molecular mass, molar absorption coefficients (ϵ), and absorption maxima (λ_{max}) of purified dyes.

The conditions and performance of immobilization reactions of the dye-ligands are summarized in Table 2. It is likely that steric effects from the bulky galactosyl moiety onto the neighbouring triazine hinder the reaction for accessing the agarose hydroxyls, as indicated by longer reaction times required for the two biomimetic ligands. All adsorbents were substituted with dye-ligand at approximately the same level (2.2–2.4 μmol dye/g moist gel). When comparing affinity adsorbents, equal ligand substitution effected by synthesis rather than dilution with unsubstituted gel is an important but often overlooked pre-requisite [13,14,16]. Furthermore, extreme levels of ligand substitution may lead to no binding, due to the steric effect caused by the large number of dye molecules, or even to non-specific protein binding [46,47].

3.3. Analysis of biomimetic dyes

The ABCD and ABX patterns of the aromatics of anthraquinone and 1,4-diamino-substituted phenyl rings, respectively, are as expected present in the ^{1}H NMR spectra of all the compounds. The pattern and assignments are based on those of the ^{1}H and ^{13}C NMR spectra of the commercially purchased reference compound VBAR.

The ^{1}H NMR spectra of BM compounds show complex, yet discernible multiplets, ranging from δ 3.50 to 4.15 ppm and δ 4.60 to 5.30 ppm, attributed to –CH–CH, –CH–NH and –CH–OH couplings, respectively.

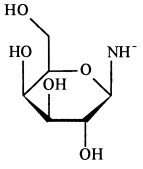
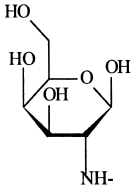
It is known that the reference compound VBAR does not exhibit a molecular ion (M^{+}) peak under EI ionization [48]. Indeed, no such ion has been observed in EI, FAB or ESI spectra. BM compounds behaved similarly. However, comparing the fragmentation pattern of BM1 with VBAR, under the

above ionization modes, allowed for the detection of fragments, resulting, most probably, from primary C–N, C–O fission.

3.4. Affinity chromatographic study of dye adsorbents with GAO

Biomimetic and non-biomimetic adsorbents were evaluated for their ability to purify GAO from *D. dendroides* extract. Agarose itself has been reported to present a certain affinity for GAO, thus being able to purify this enzyme. Therefore, preliminary experiments were executed for determining the appropriate ionic strength of the irrigating buffer, in order to exclude the interference of agarose interactions with GAO in the affinity chromatography runs. At 10 m*M* potassium phosphate buffer, pH 7.0, agarose exhibits strong binding ability for GAO (>80%), leading to moderate enzyme purification (7.8-fold) [49]. However, at 100 m*M* potassium phosphate buffer, pH 7.0, agarose does not bind GAO, so this ionic strength was chosen for the rest of this study. Table 3 shows the purifying ability and the enzyme recovery of the dye adsorbents, starting from crude untreated GAO extract, after biospecific elution (5 m*M* galactose). The two new galactosyl-biomimetic dye adsorbents (BM1 and BM2) exhibited substantially higher purifying ability for the target enzyme, compared to the non-biomimetic control adsorbent (CB3GA). Furthermore, adsorbent BM1, which bears 1-amino-1-deoxy-β-D-galactose linked to the triazine ring as terminal biomimetic moiety, displayed the highest purifying ability for GAO (13.5-fold), which is ~58% higher than the purification obtained after chromatography on agarose gel at 10 m*M* ionic strength. For the biomimetic ligands, it is likely that the chromophore portion, linked to the triazine ring, may act as a ''spacer'' that protrudes the galactosyl moiety to the solvent, thus facilitating the interaction with GAO (Fig. 1). This function is reminiscent of that of the phosphonate-biomimetic dye designed for calf intestine alkaline phosphatase [10]. In addition, it is conceivable that a better orientation of the galactosyl moiety towards the GAO binding site and a reduced steric hindrance of the polymeric support during the complex formation, may allow for a strong interaction, as observed for BM1 with GAO even in the presence of 100 m*M* buffer. The above

Table 3
Affinity chromatography evaluation of dye adsorbents for binding GAO activity

Dye-ligand (-R)	Specific activity (U/mg)	Purification (-fold)	Recovery (%)
BM1 	689	13.5	85
BM2 	316	6.2	73
CB3GA -*o*-HN*Benz*SO_3^-	179	3.5	26

features are probably not met successfully by agarose gel when this is used in GAO purification.

The purified enzyme, after affinity chromatography on BM1, exhibits at least twice as high specific activity, as compared to the enzyme purified on melibiose-polyacrylamide affinity column [50]. Biomimetic adsorbent BM2 displayed a purifying ability of 6.2-fold, which is somewhat below the purification obtained on agarose gel in 10 m*M* potassium phosphate buffer, pH 7.0. The reduced amount of enzyme bound on BM2 and the lower purification factor obtained, can both be explained by the fact that, although the specifically-binding moieties of BM1 and BM2 are predicted to be identical, the experimental findings may have an indirect origin: differential competition for binding to these two biomimetics by other proteins. Several other cellular proteins bind to galactose, including dehydrogenases, kinases and disaccharidases [51]. Since these enzymes have different modes of galactose binding, they may be able to compete more effectively with GAO for binding to the BM2 column than to the BM1 column. The relatively limited size of the GAO-interacting part of BM1 and BM2—essentially the galactose moiety alone—would facilitate competition for binding by other proteins in the cell extract. This binding, mainly through the galactose portion, also explains the lack of effectiveness of CB3GA itself. The limited binding of GAO to the CB3GA column presumably arises from non-specific interactions with the enzyme, which are more effective with other proteins of the extract.

The new biomimetic dye adsorbent BM1, even when used in a single-step purification procedure, leads to GAO of acceptable specific activity (689 U/mg) and good recovery (85%). However, as will be shown later in Section 3.6 and Table 6, by inserting a preliminary ion-exchange chromatography step, followed by affinity chromatography on BM1, one may obtain GAO of the highest specific activity ever reported.

3.5. Dissociation constant (K_D) and GAO-binding capacity of immobilized BM1 ligand

Since immobilized BM1 ligand was proved to be the best affinity chromatography ligand for GAO, it was further examined. Fig. 2 shows the elution

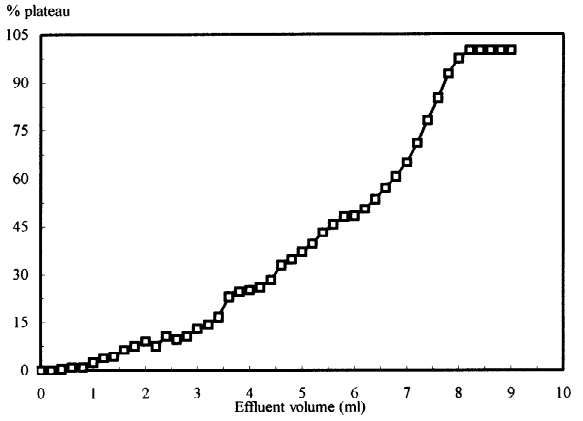


Fig. 2. Analytical affinity chromatography of *D. dendroides* GAO for the determination of the K_D value of immobilized BM1–GAO complex. The chromatography data collected are substituted in Eq. (2) (V_0 = 0.2 ml) and the results obtained are shown in Table 4.

profile for an analytical chromatography run to determine the K_D value, obtained by plotting relative eluting GAO activity versus effluent volume; this profile is composed of an elution front and a plateau. The elution volume, *V*, of the front of the enzyme approximately equals the volume of effluent corresponding to the half-enzyme concentration in the starting material or at the plateau, $[E]_0/2$. In case the elution front of the enzyme is not symmetrical with regard to the midpoint, then, if fractions of constant volume are collected, *V* should be calculated from Eq. (1) [35,36]:

$$V = an - a\sum_{i=1}^{n}\frac{[E]_i}{[E]_0} \quad (1)$$

where *a* is the fraction volume, *n* is the number of a plateau fraction, and $[E]_i$ is the enzyme concentration in fraction *i*. Because *V* can be determined from Eq. (1), the K_D value is then calculated from Eq. (2) [35,36]:

$$K_D = \frac{L}{V - V_0} \quad (2)$$

where *L* is the amount of the immobilized dye-ligand, and V_0 is the elution volume of a substance which does not interact with the immobilized ligand (void volume).

The K_D value for the immobilized ligand–GAO

Table 4
Data obtained from analytical affinity chromatography of GAO (pH 7.0, 4 °C) and used in Eq. (2) for the determination of the dissociation constant K_D ($V_0 = 0.2$ ml)

Immobilized ligand	Adsorbent quantity (mg)	L (μmol)	V (ml)	K_D (μM)
BM1	100	0.24	5.4	45.8

complex is equal to 45.8 μM (Table 4). From the profile shown in Fig. 2, the capacity of the BM1 adsorbent is also calculated, and is equal to 113 U/0.16 ml adsorbent (709 U/ml adsorbent).

3.6. The purification of GAO from D. dendroides extract on immobilized BM1 ligand

Even though one-step chromatography of GAO on BM1 led to satisfactory results concerning purification and recovery of the enzyme (Section 3.4, Table 3), further efforts were made in order to achieve an even higher specific activity, since a high specific activity was recently reported (1400 U/mg for rGAO) [52]. In general, ion-exchange chromatography has proved to be a useful tool in improving purification when it is added as a preliminary step to the purification procedure [12,14].

Prior to the design of the purification protocol for GAO, initially the ion-exchange chromatography conditions were optimized. The influence of pH and the nature of the ion exchanger on enzyme purification were investigated (Table 5). At pH 6.0 the enzyme recovery from the CM column was reduced to 65%, and at pH 6.5 the CM column displayed maximum purifying ability (4.8-fold) together with full recovery, whereas considerable fall is observed for the rest of the conditions applied. This can be easily explained, given the fact that the isoelectric point (pI) value of GAO from *D. dendroides* has been reported to be ~12 [53]. At pH 6.5, GAO is strongly positively charged, while the impurities present in the extract may have such pI values, that either do not bind, or bind in a much weaker mode. The DEAE column did not bind the enzyme at pH range 6.5–7.5, and only at pH 8.0 the column bound ~30% of the applied GAO activity. Therefore, the CM cation exchanger and the pH value of 6.5 were finally chosen for the first step of the purification protocol.

Table 5
Effect of the ion-exchanger nature and pH on the purification of GAO from *D. dendroides*

Ion exchanger	pH	Purification (-fold)
CM	6.0	4.5
CM	6.5	4.8
CM	7.0	2.7
CM	7.5	3.0
DEAE	6.5	2.1
DEAE	7.0	3.3
DEAE	7.5	3.5
DEAE	8.0	3.5

The purification of GAO for the DEAE column was measured in the throughput fraction.

For the second step of the purification protocol, employing affinity chromatography on immobilized BM1 ligand, the conditions adopted were those determined from the affinity chromatographic study, as described in Section 3.4.

The results of a typical purification run, based on the two-step procedure, are summarized in Table 6. The specific activity of the purified GAO is equal to 2038 U/mg, which is the highest ever reported for this enzyme (see below). The purity of the enzyme preparation was analyzed by SDS–PAGE, and showed the presence of a single band, after Coomassie Blue R-250 staining (Fig. 3).

Galactose oxidase has been purified from commercial samples using affinity chromatography on Sepharose 6B by other workers [49]. This procedure led to a specific activity of 255 or 263 U/mg (after modifying enzymatic units from the peroxidase/*o*-dianisidine assay to the peroxidase/*o*-toluidine assay; conversion ratio 1:1.19) and 90% overall recovery. A later purification method for GAO from *D. dendroides* [54] included two stages in a time consuming procedure (2–3 days), but it was not based on affinity chromatography. Another procedure for GAO purification from *D. dendroides* included thermal treatment, ammonium sulphate fractionation and use of Sepharose 6B column [55]. This procedure led to a specific activity of 116 U/mg and 64% overall recovery. A more recent purification procedure for *D. dendroides* GAO employed affinity chromatog-

Table 6
Purification protocol of GAO from *D. dendroides* extract[a]

Step	Volume (ml)	Activity (U)	Protein (mg)	Specific activity (U/mg)	Purification (-fold)	Recovery (%)
Crude extract	1.15	21.6	0.42	51.4	1	100
Cation-exchange chromatography (elution with 120 m*M* KCl)	7	18.6	0.075	248	4.8	86.1
BM1 affinity chromatography (elution with 5 m*M* galactose)	6	15.9	0.0078	2038	39.7	73.6

[a] Procedures were performed at 4 °C. For details see text.

raphy on melibiose-polyacrylamide column, after ammonium sulphate fractionation and chromatography on a microcrystalline cellulose column [50]. This procedure led to a specific activity of 328 U/mg and 90% overall yield at the stage of the affinity column. Finally, Whittaker and Whittaker [52] have reported a two-step purification procedure of recombinant GAO expressed by *Pichia pastoris*, leading to a specific activity of 1400 U/mg and 70% overall recovery.

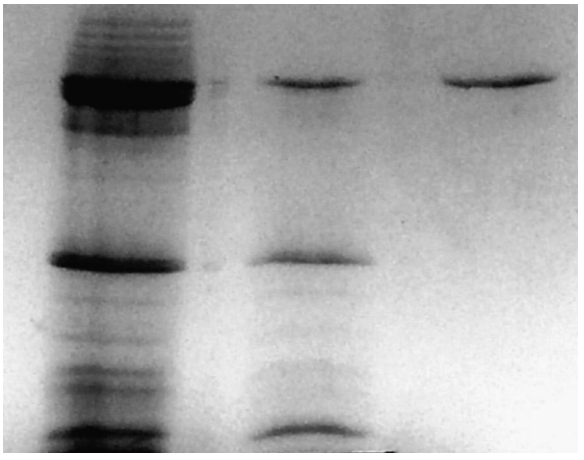


Fig. 3. SDS–PAGE analysis. Performed on a 0.75-mm-thick vertical slab gel containing 12.5% (w/v) polyacrylamide (running gel) and 2.5% (w/v) stacking gel. The protein bands were stained with Coomassie Blue R-250. Left lane: *D. dendroides* extract (15 μg total protein); middle lane: after CM-Sepharose cation-exchange chromatography (10 μg total protein); right lane: after affinity chromatography on BM1 (7.5 μg total protein).

Table 7 summarizes the results of control experiments with regard to the selectivity of the BM1 affinity adsorbent for interacting (binding) with enzymes other than GAO, when run under the same conditions as applied for GAO. None of the enzymes tested have shown any significant binding to the affinity adsorbent. This finding strengthens the view that the BM1 ligand acts as a true affinity ligand for GAO.

In conclusion, in this report we describe the design, synthesis and application of new galactosyl-biomimetic ligands and respective adsorbents for the affinity purification of galactose oxidase. The affinity purification method described is simple and efficient, as it yields pure enzyme of the highest specific activity reported so far and with good recovery.

Table 7
Control experiments concerning the binding selectivity of immobilized BM1[a]

Enzyme	Source	Units applied	Bound enzyme (%)
GalDH	*P. fluorescens*	0.3	0.2
GOD	*A. niger*	16.8	0.1
GDH	*P. fluorescens*	0.1	0.8
GDH	Yeast	0.5	0.6
ADH	Yeast	2.2	4.3
ADH	Green peas	0.3	9.2

[a] On the affinity column (0.5 ml), previously equilibrated with 100 m*M* potassium phosphate buffer, pH 7.0, were applied the units of the enzymes shown that had been dialyzed in the same equilibration buffer as above (4 °C). After the adsorbent was washed with equilibration buffer, elution of bound proteins was effected using 1 *M* KCl.

4. Nomenclature

ADH	Alcohol dehydrogenase
BM	Biomimetic ligand
CB3GA	Cibacron Blue 3GA
GalDH	Galactose dehydrogenase
GAO	Galactose oxidase
GDH	Glucose dehydrogenase
GOD	Glucose oxidase
VBAR	Vilmafix Blue A-R

References

[1] P. Cuatrecasas, J. Biol. Chem. 245 (1970) 3059.
[2] S.R. Narayanan, L.J. Crane, Trends Biotechnol. 8 (1990) 12.
[3] N.E. Labrou, Y.D. Clonis, J. Biotechnol. 36 (1994) 95.
[4] C. Jones, A. Patel, S. Griffin, J. Martin, P. Young, K. O'Donnell, C. Silverman, T. Porter, I. Chaiken, J. Chromatogr. A 707 (1995) 3.
[5] G.J. Sofer, J. Chromatogr. A 707 (1995) 23.
[6] Y.D. Clonis, T. Atkinson, S. Burton, C. Lowe (Eds.), Reactive Dyes in Protein and Enzyme Technology, Macmillan, Basingstoke, 1987.
[7] C.R. Lowe, J.C. Pearson, Methods Enzymol. 104 (1983) 97.
[8] Y.D. Clonis, N.E. Labrou, V.Ph. Kotsira, C. Mazitsos, S. Melissis, G. Gogolas, J. Chromatogr. A 891 (2000) 33.
[9] N.E. Labrou, Y.D. Clonis, in: M.A. Vijayalakshmi (Ed.), Theory and Practice of Biochromatography, Taylor and Francis, London, 2002, pp. 235–251, chapter 8..
[10] N.M. Lindner, R. Jeffcoat, C.R. Lowe, J. Chromatogr. 473 (1989) 227.
[11] Y.D. Clonis, C.V. Stead, C.R. Lowe, Biotechnol. Bioeng. 30 (1987) 621.
[12] N.E. Labrou, E. Eliopoulos, Y.D. Clonis, Biotechnol. Bioeng. 63 (1999) 322.
[13] N.E. Labrou, A. Karagouni, Y.D. Clonis, Biotechnol. Bioeng. 48 (1995) 278.
[14] S.C. Melissis, D.J. Rigden, Y.D. Clonis, J. Chromatogr. A 917 (2001) 29.
[15] N.E. Labrou, E. Eliopoulos, Y.D. Clonis, Biochem. J. 315 (1996) 695.
[16] N.E. Labrou, Y.D. Clonis, J. Biotechnol. 45 (1995) 185.
[17] S.J. Burton, C.V. Stead, C.R. Lowe, J. Chromatogr. 455 (1988) 201.
[18] K. Sproule, P. Morrill, J.C. Pearson, S.J. Burton, K.R. Hejnes, H. Valore, S. Ludvigsen, C.R. Lowe, J. Chromatogr. B 740 (2000) 17.
[19] S.T. Teng, K. Sproule, A. Husain, C.R. Lowe, J. Chromatogr. B 740 (2000) 1.
[20] C.S. Frings, H.L. Pardue, Anal. Chem. 36 (1964) 2477.
[21] D. Macmillan, C.R. Bertozzi, Tetrahedron 56 (2000) 9515.
[22] S.M. Partridge, Nature 158 (1947) 270.
[23] S.M. Partridge, Biochem. J. 42 (1948) 238.
[24] S. Burton, S. McLoughlin, C.V. Stead, C.R. Lowe, J. Chromatogr. 435 (1988) 127.
[25] N.E. Labrou, Y.D. Clonis, Arch. Biochem. Biophys. 316 (1995) 169.
[26] G. Tsamadis, N. Papageorgakopoulou, Y.D. Clonis, Bioprocess Eng. 7 (1992) 213.
[27] K. Yamamoto, Y. Kondo, H. Kumagai, T. Tochikura, Agric. Biol. Chem. 49 (1985) 2463.
[28] K. Wallenfels, G. Kurz, Biochem. Z. 335 (1962) 559.
[29] H.U. Bergmeyer, K. Gawehn, M. Grassl, in: H.U. Bergmeyer (Ed.), 2nd ed., Methods of Enzymatic Analysis, Vol. I, Academic Press, New York, 1974, p. 457.
[30] H.J. Strecker, Methods Enzymol. 1 (1955) 335.
[31] B.L. Vallee, F.L. Hoch, Proc. Natl. Acad. Sci. USA 41 (1955) 327.
[32] M. Bradford, Anal. Biochem. 72 (1976) 248.
[33] J.C. Bearden Jr., Biochim. Biophys. Acta 533 (1978) 525.
[34] U.K. Laemmli, Nature (London) 227 (1970) 680.
[35] K. Kasai, S. Ishii, J. Biochem. 77 (1975) 261.
[36] K. Kasai, Y. Oda, M. Nishikata, S. Ishii, J. Chromatogr. 376 (1986) 33.
[37] N. Ito, S.E. Phillips, C. Stevens, Z.B. Ogel, M.J. McPherson, J.N. Keen, K.D. Yadav, P.F. Knowles, Nature 350 (1991) 87.
[38] H.M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T.N. Bhat, H. Weissig, I.N. Shindyalov, P.E. Bourne, Nucleic Acids Res. 28 (2000) 235.
[39] G.J. Kleywegt, T.A. Jones, Acta Crystallogr. D54 (1998) 1119.
[40] T.A. Jones, J. Zou, S.W. Cowan, M. Kjeldegaard, Acta Crystallogr. A 47 (1991) 110.
[41] J.J. Kraulis, Appl. Cryst. 24 (1991) 946.
[42] N. Ito, S. Phillips, K. Yadav, P. Knowles, J. Mol. Biol. 238 (1994) 794.
[43] J.-F. Biellmann, J.-P. Samama, C.J. Bränden, H. Eklund, Eur. J. Biochem. 102 (1979) 107.
[44] C. Lowe, D. Small, A. Atkinson, Int. J. Biochem. 13 (1981) 3.
[45] D. Hanggi, P. Carr, Anal. Biochem. 149 (1985) 91.
[46] Y. Liu, E. Stellwagen, J. Chromatogr. 376 (1986) 149.
[47] Y. Liu, E. Stellwagen, J. Biol. Chem. 262 (1987) 583.
[48] T. Prestera, H.J. Prochaska, P. Talalay, Biochemistry 31 (1992) 824.
[49] M.W.C. Hatton, E. Regoeczi, Biochim. Biophys. Acta 438 (1976) 339.
[50] F.M. Kelleher, S.B. Dubbs, V.P. Bhavanandan, Arch. Biochem. Biophys. 263 (1988) 349.
[51] A. Bairoch, Nucleic Acids Res. 28 (2000) 304.
[52] M.M. Whittaker, J.W. Whittaker, Protein Expr. Purif. 20 (2000) 105.
[53] D.J. Kosman, M.J. Ettinger, R.E. Weiner, E.J. Massaro, Arch. Biochem. Biophys. 165 (1974) 456.
[54] P. Tressel, D. Kosman, Anal. Biochem. 105 (1980) 150.
[55] M.H. Mendonça, G.T. Zancan, Arch. Biochem. Biophys. 252 (1987) 507.