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AGAROSE-LINKED XANTHOSINE: A BIOSPECIFIC RESIN FOR GUANINE AMINOHYDROLASE

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ABSTRACT

Polymer-bound xanthosine (**4**) has been prepared. Condensation of xanthosine with ethyl 4-oxovalerate and saponification of the product gave 2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]xanthosine. The latter was coupled to 6-aminohexylagarose through its carboxylic group, to yield the polymer **4**. The content of bound ligand was 8 μmol/g of moist gel, a value that agrees with the number of free amino groups determined by the trinitrobenzenesulfonic acid assay before coupling. Immobilised xanthosine was used as a biospecific resin (inhibitor resin) for guanine aminohydrolase (EC 3.5.4.3), to separate the enzyme from a mixture containing adenosine deaminase (EC 3.5.4.4).

INTRODUCTION

The biospecificity of affinity resins bearing 2′,3′-*O*-ethylidenenucleosides towards nucleobase-converting enzymes has prompted the design of a xanthosine-resin specific for guanine aminohydrolase (inhibitor resin)[1–5].

Guanine aminohydrolase (GAH, guanase, EC 3.5.4.3, mol. wt. 55,000) catalyses the deamination of guanine to ammonia and xanthine, which is oxidised *in vivo* to uric acid by xanthine oxidase; the enzyme has been isolated from several natural sources[6,7].

The affinity resin now described should allow an easy separation of guanase from such other deaminases as adenosine deaminase (ADA, EC 3.5.4.4) or cytidine deaminase (CDA, EC 3.5.4.5). In addition, it may prove useful for the preparation of highly purified enzyme from crude preparations.

RESULTS AND DISCUSSION

The reaction of xanthosine (**1**) with ethyl 4-oxovalerate (levulinate) in the presence of hydrochloric acid and triethyl orthoformate gave 2′,3′-*O*-[1-(2-ethoxy-

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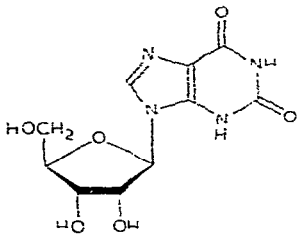

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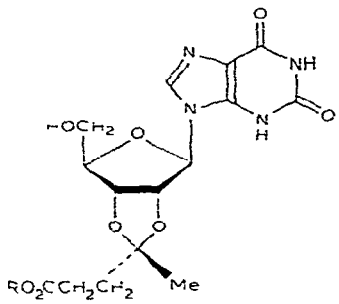


2a R = Et
2b R = H

carbonylethyl)ethylidene]xanthosine (**2a**) in 46% yield. The reaction succeeds only if thoroughly dried starting-material is used, because traces of water cause hydrolysis of the N–glycosyl bond.

The ^{13}C- and ^{1}H-n.m.r. spectra of **2a** show that the acetalation occurs at HO-2′,3′ and that only the *R* diastereoisomer is formed[8]. This is in contrast to acetalation reactions with unsymmetrical ketones, where both isomers are formed[9].

Alkaline hydrolysis of **2a** gave the acid **2b**, which was obtained as its sodium salt due to the acidity of the xanthine moiety. Chromatography on Dowex 1X2 anion-exchange resin (acetate form) and elution with a linear gradient of 0.5M acetic acid–water yielded pure **2b**.

To show that xanthosine as well as the acid **2b** are bound to the active site of guanine aminohydrolase, they were tested as inhibitors in the enzymic deamination reaction. The enzymic assay depends upon the difference in the λ_{max} of guanine (246 nm) compared with that of xanthine (267 nm). By following the decrease in the absorbance at 246 nm, it was shown that the relative, initial velocities of the enzymic deamination decline to 60% at a molar ratio of guanine to xanthosine of 1:8.5 and

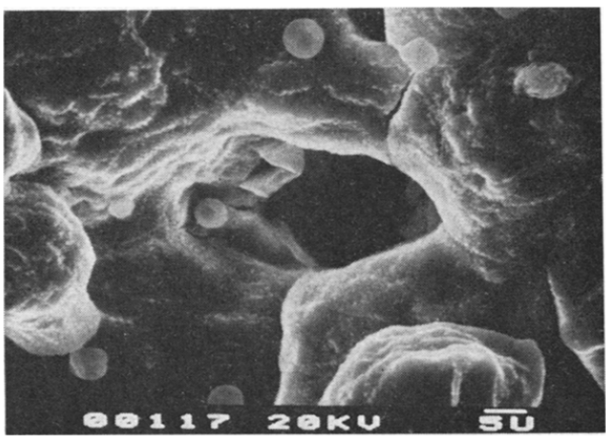


Fig. 1. REM-microphotograph of 6-aminohexylagarose (3); calibration: 5 μm.

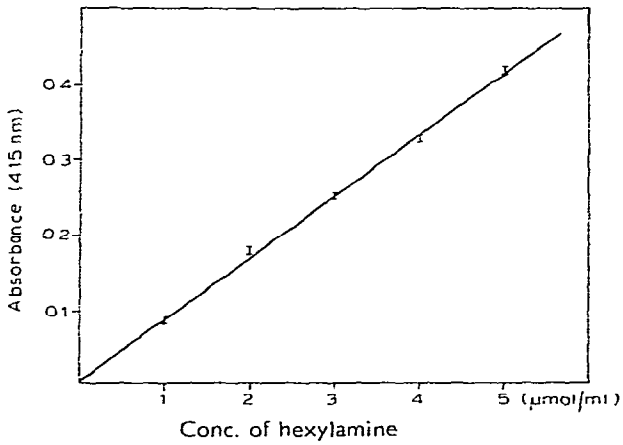
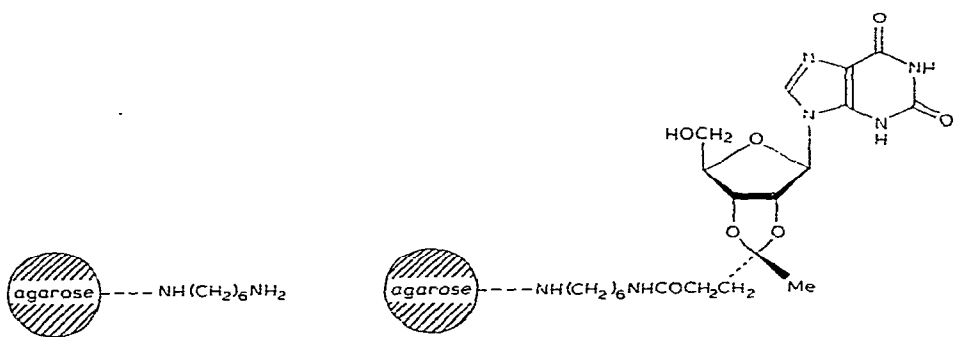


Fig. 2. Standard curve showing A_{415} of the dye *versus* increasing amounts of hexylamine. For details, see Experimental.



3 4

to 65% at a molar ratio of guanine to the acid **2b** of 1:8.5. Thus, both the nucleoside and the acid **2b** are inhibitors of guanase, and the latter can therefore be used as a ligand in biospecific resins for this enzyme.

It has been shown that agarose is a suitable polymer-matrix for biospecific ligands[10]. Its macroporous structure allows even the diffusion of macromolecules[11,12]. Agarose can be activated with BrCN and then converted into the 6-aminohexyl derivative **3** by treatment with 1,6-hexanediamine[13]. As can be seen from Fig. 1, the macroporous and spongy structure is maintained even after the cross-linking that accompanies the activation reaction.

For determination of the concentration of primary amino groups, the qualitative 2,4,6-trinitrobenzenesulfonic acid (TNBS) method[14] was quantified. A standard curve (Fig. 2) was derived, using hexylamine, and the concentration of primary amino groups in the polymer **3** was determined after solubilisation (2M HCl) of the TNBS-stained gel. The 6-aminohexylagarose contained 9 ± 1 μmol of primary amino groups/g of moist gel.

The acid **2b** was coupled with **3** in *p*-dioxane–water in the presence of a water-soluble carbodiimide for 24 h at room temperature. The content of bound ligand in the dry derivative **4**, determined on the basis of the u.v. absorbance after hydrolysis, was 200 μmol/g. For the moist gel containing 96% of water, this value corresponds to 8.0 μmol/g of **4**, and is identical to the number of free amino groups before coupling, demonstrating the completeness of the coupling reaction.

The chromatographic behaviour of guanine aminohydrolase on the inhibitor-resin **4** confirmed expectations. Whereas the enzyme was eluted from 6-aminohexyl-agarose (**3**) within one void volume of starting buffer (0.07M phosphate buffer, pH 7.6; Fig. 3*a*), 0.5M sodium chloride was required to elute the enzyme from **4** (Fig. 3*b*).

When mixtures of guanine aminohydrolase and adenosine deaminase were chromatographed on **4**, the latter enzyme was completely eluted with the void volume of buffer (0.07M phosphate buffer, pH 7.6), whereas guanine aminohydrolase required 0.5M sodium chloride (Fig. 3*c*). The purity of the enzymes was determined by SDS-gel electrophoresis[15] (Fig. 4), using a 13% polyacrylamide gel.

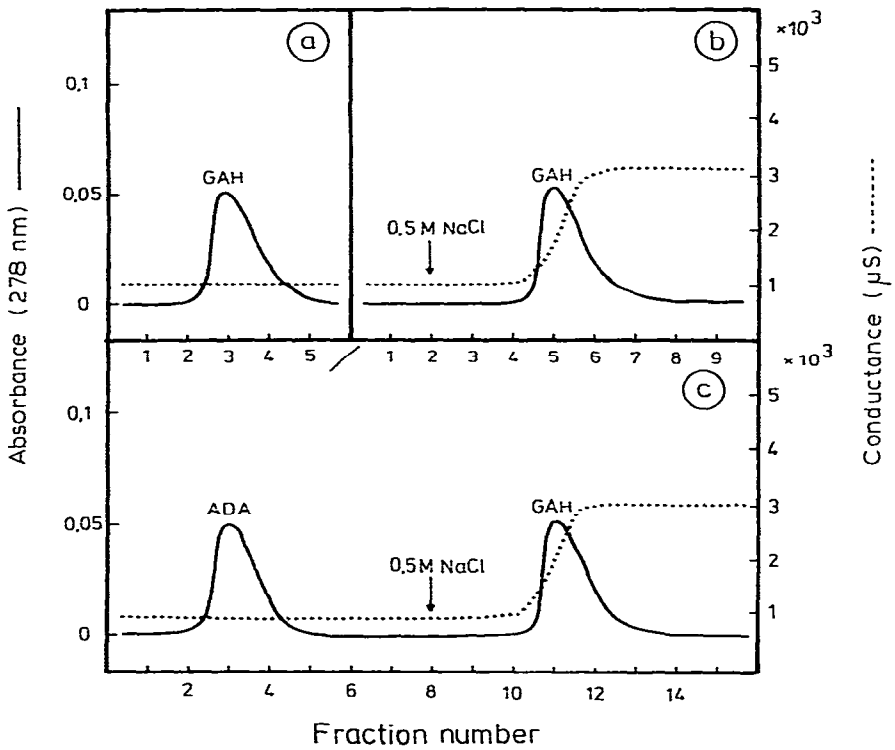


Fig. 3. (a) Chromatography of guanine aminohydrolase (GAH) on the resin **3**, (b) affinity chromatography of GAH (1 mg/100 μl) on **4**, and (c) of GAH in the presence of adenosine deaminase (ADA, 1 mg/100 μl). The enzymes were applied to a column (10 × 0.9 cm) equilibrated with 0.07M phosphate buffer (pH 7.6) and eluted with 0.5M sodium chloride.

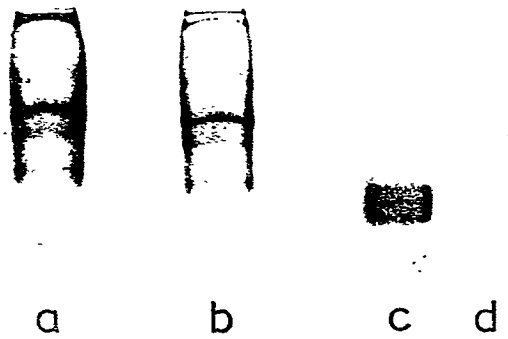


Fig. 4. SDS–Polyacrylamide-gel electrophoresis of (a) GAH, (b) the content of the main peak, (c) ADA, and (d) the peak content of the pre-zone of the elution profile. For details, see Experimental section.

EXPERIMENTAL

General. — Melting points were determined on a Büchi SMP 20 apparatus and are not corrected. The ^{1}H- and ^{13}C-n.m.r. spectra were recorded for solutions in $(CD_3)_2SO$ (internal Me_4Si) with a Bruker HX-60 or a WP-270 spectrometer, and chemical shifts are reported in δ values. U.v. spectra were recorded with Zeiss PMQ 3 or Shimadzu UV-200 spectrometers, using 1-cm cuvettes. Microanalyses were performed by Mikroanalytisches Labor Beller, Göttingen, Germany.

Column chromatography was performed on Dowex 1-X2 (AcO^-) resin (200–400 mesh), using an LKB UltroRac fraction-collector with an LKB Uvicord II as detection unit. T.l.c. was performed on silica gel F-254 (Woelm) with *A*, chloroform–methanol (8:2); and *B*, 0.25M LiCl. Affinity chromatography was performed in thermostatted, jacketed, Multichrom columns (30 × 0.9 cm, Serva-Technik, Heidelberg, Germany) attached to an LKB Uvicord III photometer (at 254 and 278 nm). In addition, the affinity column was attached to a conductivity cell (with a cell constant K = 12.6 cm^{-1}; WTW Instruments, Weilheim, Germany) on which conductance was constantly measured. Fractions were collected with an LKB UltroRac fraction-collector. The flow rate (24 ml/h) was adjusted with an LKB Vario Perpex pump II.

6-Aminohexylagarose (AH-Sepharose 4B, 3), xanthosine, guanine aminohydrolase (EC 3.5.4.3), and adenosine deaminase (EC 3.5.4.4) were commercial products.

Relative, initial velocities of the deamination of guanine by guanine aminohydrolase in the presence of various inhibitors were determined by the addition of guanine aminohydrolase (5×10^{-3} units) to 1 ml of 0.07M phosphate buffer (pH 7.6) containing 0.1 μmol of guanine and 0.85 μmol of the inhibitor. The decrease of guanine at room temperature was followed at 246 nm for 10 min. The slope between

the first and second minute was defined as the initial velocity, and arbitrarily taken as 100 for guanine.

Sodium dodecyl sulfate (SDS)-gel electrophoresis was performed according to the method of Weber and Osborn[15], using a 13% polyacrylamide gel and a Desaga apparatus.

2',3'-O-[1-(2-Ethoxycarbonylethyl)ethylidene]xanthosine (**2a**). — To a suspension of xanthosine (5.7 g, 20 mmol; dried for 24 h at 50° *in vacuo*) in dry *N,N*-dimethylformamide (100 ml) were added ethyl levulinate (5.76 g, 40 mmol) and triethyl orthoformate (5 ml, 30 mmol); insoluble material was dissolved by adding a 7M solution (10 ml) of hydrogen chloride in dry *p*-dioxane. The mixture was kept for 24 h at room temperature and then poured into ether. The upper layer was decanted, and the oily residue was washed twice with ether and then crystallised by adding ethyl acetate. Recrystallisation from methanol–ethyl acetate gave 3.83 g (46.5%) of colorless **2a**, m.p. 198–200°, R_F 0.81 (solvent *A*); λ_{max}^{MeOH} 241 and 257 nm (ε 7600 and 8100). ^{1}H-N.m.r. data: δ 7.95 (s, H-8), 6.02 (d, *J* 3 Hz, H-1'), 5.05 (m, H-2',3'), 4.10 (m, H-4'), 4.06 (q, *J* 7 Hz, ester CH_2), 3.50 (m, H-5'), 2.30 (m, CH_2-C=O), 2.10 (m, acetal CH_2), 1.35 (s, acetal Me), and 1.20 (t, *J* 7 Hz, ester Me). ^{13}C-N.m.r. data: δ 172.58 (C=O), 157.82 (C-2), 150.63 (C-4), 139.88, 134.70 (C-6 hydrate and C-8), 115.98 (C-5), 114.17 (acetal C), 89.24 (C-1'), 85.81 (C-4'), 83.09 (C-3'), 80.82 (C-2'), 61.13 (C-5'), 59.84 (O-CH_2), 33.55 (CH_2-C=O), 28.24 (acetal CH_2), 23.38 (acetal Me), and 13.99 (ester Me).

Anal. Calc. for $C_{17}H_{22}N_4O_8$: C, 49.75; H, 5.40; N, 13.65. Found: C, 49.66; H, 5.38; N, 13.48.

2',3'-O-[1-(2-Carboxyethyl)ethylidene]xanthosine (**2b**). — A solution of **2a** (2.0 g, 4.87 mmol) in a 1:1 mixture (60 ml) of ethanol and M sodium hydroxide was kept for 30 min at room temperature and then neutralised (glass electrode) with Amberlite IR-120 (H^+) resin, filtered, and evaporated to dryness. The residue was dissolved in water (50 ml) and, after adding a few drops of conc. ammonia, chromatographed on a column (30 × 1.5 cm) of Dowex 1X2 (OAc^-) resin with a linear gradient of 0.5M acetic acid–water (500 ml). The acid **2b** was found in the main peak. Appropriate fractions were combined and evaporated to dryness, giving 1.28 g (68.7%) of colorless **2b**, R_F 0.63 (solvent *B*); λ_{max}^{MeOH} 253 nm (ε 7800). ^{1}H-N.m.r. data: δ 8.02 (s, H-8), 6.05 (d, *J* 3 Hz, H-1'), 5.1 (m, H-2',3'), 4.3 (m, H-4'), 3.56 m, H-5'), 2.3 (m, aliphatic CH_2), and 1.35 (s, acetal Me).

Anal. Calc. for $C_{15}H_{18}N_4O_8$: C, 47.12; H, 4.75; N, 14.66. Found: C, 47.42; H, 4.92; N, 14.91.

Coupling reaction of 6-aminohexylagarose (**3**). — To a solution of **2b** (50 mg, 0.13 mmol) in water (10 ml) was added **3** (5 ml; content of primary amino groups, 9 ±1 μmol/g of moist gel) at pH 6. The suspension was shaken for 10 min and 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride (100 mg, 0.52 mmol) was added. Shaking was continued overnight, and the product was collected, washed with 0.1M sodium hydrogencarbonate (250 ml), 0.5M sodium chloride (250 ml), and water, to give the coupled derivative **4** (5 g).

Portions (0.5 g) of moist **4** and agarose **3** were dried for 72 h at 95° *in vacuo* to constant weight. The dry powders **4** and **3** (6.0 mg, each) and **2b** (1.25 μmol) were hydrolysed separately with 0.5M hydrochloric acid (5 ml) for 30 min at 100°. The absorbances at 253 nm (1-cm path-length) were 0.340, 0.173, and 0.170, respectively. Thus, dry **4** contains 200.0 μmol of ligand/g. Assuming a water content of 96%, the moist gel **4** contains 8 μmol of ligand/g.

Quantitative determination of the primary amino groups of 6-aminohexylagarose (**3**). — (*a*) For a standard curve, the following pipetting procedure was performed: aliquots (1, 2, 3, 4, and 5 ml) of a stock solution of hexylamine (1 μmol/ml) were mixed with saturated, aqueous sodium tetraborate (1 ml) and brought to a total volume of 6 ml with water. After addition of 3% aqueous 2,4,6-trinitrobenzenesulfonic acid (100 μl), the reaction mixture was incubated at 37° for 30 min. Then 2M hydrochloric acid (4 ml) and acetone (5 ml) were added. After 3-fold dilution with acetone–water (1 : 1), the absorbance was read at 415 nm in a 1-cm quartz cuvette.

(*b*) A portion of **3** (250 mg) was suspended in saturated, aqueous sodium tetraborate (1 ml) and treated as described above. After addition of 2M hydrochloric acid (4 ml), the gel was solubilised at 100°. After addition of acetone (5 ml) and 3-fold dilution with water–acetone (1 : 1), the absorbance was read at 415 nm. The concentration of primary amino groups of commercial 6-aminohexylagarose (**3**) was found to be 9 ± 1 μmol/g of moist gel.

Affinity chromatography of guanine aminohydrolase on **4**. — The gel **4** was thoroughly washed with water, packed into a jacketed column (10 × 0.9 cm), thermostatted at 20.0°, and equilibrated with 2–3 void volumes of phosphate buffer (0.07M, pH 7.6). Before each chromatographic run, 100 μl of an ammonium sulfate-suspension of guanine aminohydrolase (10 mg/ml) were diluted with 5 ml of water and then concentrated in an Amicon-B-15, Macrosolute Concentrator to a final volume of 100 μl. The enzyme solution was then applied to the resin **4**, or first mixed with 100 μl of a stock solution of adenosine deaminase (10 mg/2 ml) and then injected onto the column. Conductance and A_{278} were constantly measured and 2-ml fractions were collected. Adenosine deaminase was eluted with the void volume of buffer and did not contain any guanine aminohydrolase activity: this could be demonstrated by qualitative t.l.c. after incubating guanine with the contents of the breakthrough peak for 1 h at 37° in phosphate buffer (0.07M, pH 7.6). Guanine aminohydrolase was eluted from **4** with the void volume on using 0.5M sodium chloride. The peak contents were also confirmed by SDS-gel electrophoresis (Fig. 4).

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