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IMMOBILISATION OF TUBERCIDIN AND NEBULARINE *via* 2′,3′-CYCLIC ACETALS: BIOSPECIFIC AND NON-SPECIFIC INTERACTION OF AFFINITY RESINS WITH ADENOSINE DEAMINASE

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ABSTRACT

Polymer-bound tubercidin (**7a**) and nebularine (**7c**) have been prepared. Condensation of tubercidin with ethyl levulinate and saponification of the product gave 2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]tubercidin (**3b**). The corresponding reaction with nebularine failed, but application of the reaction sequence to 6-thioinosine gave 2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]-6-thioinosine (**4b**), which was desulphurated to give 2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]nebularine (**5b**). The acids **3b**, **4b**, and **5b** were coupled to 6-aminohexylagarose through their carboxyl groups, to yield the polymers **7a–c**. Stronger binding of adenosine deaminase to the inhibitor gel **7c** was observed than when the polymer bearing adenosine as ligand was used as an affinity resin. The non-inhibitor gel **7a** showed only weak binding, which is equivalent to the non-biospecificity of polymers of type **7**.

INTRODUCTION

We have reported the immobilisation of adenosine[1] and inosine by attachment of their 2′,3′-cyclic acetal derivatives to 6-aminohexylagarose and demonstrated the high specificity of these substrate- and product-affinity resins[2] for adenosine deaminase[3,4].

A problem with the highly specific adenosine (substrate) resin was its slow but continuous conversion into the inosine (product) resin, accompanied by a decrease in the affinity for this enzyme. To overcome this problem, we have investigated a ligand, nebularine[5,6] (**2b**), which cannot be deaminated enzymically, but is strongly bound to adenosine deaminase[7].

Biospecific affinity resins for adenosine deaminase may also show non-specific[8] binding to the enzyme due to hydrophobic[9,10] or ionic interaction[11] between the

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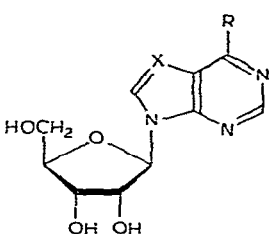
spacer and the bound protein. In order to investigate this problem, the geometry of the substrate resin was mimicked by covalent attachment to aminohexylagarose of tubercidin (7-deazaadenosine)[12,13], which is neither a substrate nor inhibitor for adenosine deaminase[14]. The resulting polymer should show only non-specific binding.

RESULTS AND DISCUSSION

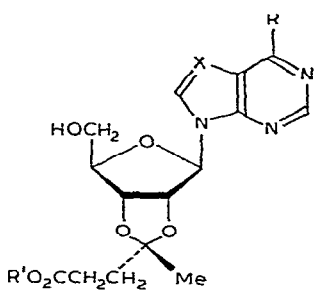
The reaction of tubercidin (**1**) with ethyl levulinate in the presence of hydrochloric acid and ethyl orthoformate gave 82% of 2′,3′-*O*-[1-(2-ethoxycarbonylethyl)-ethylidene]tubercidin. Following similar treatment of nebularine, the product could not be isolated, because of the ready hydrolysis of the *N*-glycosyl bond[15]. Since nebularine (**2b**) can be prepared by catalytic hydrogenation[7] of 6-thioinosine (**2a**), the condensation reaction was applied to the latter compound, and the acetal **4a** was isolated crystalline. Treatment of **4a** with Raney nickel effected desulphuration and gave 65% of **5a**. Attempts to hydrolyse the ester **5a** under alkaline conditions failed, because of the sensitivity of the nucleoside towards base.

In contrast to the nebularine ester **5a**, the mercapto-ester **4a** could be easily saponified, and **4b** was isolated crystalline. Treatment of **4b** with Raney nickel then yielded amorphous **5b**. Cyclic acetal formation generates a new chiral centre, and diastereoisomers are expected. From the ^{1}H- and ^{13}C-n.m.r. data, it was evident that the formation of cyclic acetals from tubercidin, 6-thioinosine, and nebularine gave only one diastereoisomer. An X-ray structure analysis[16] of 2′,3′-*O*-[1-(2-carboxyethyl)ethylidene]adenosine showed that the methyl and carboxyethyl groups were *exo* and *endo*, respectively. The similarity of the ^{13}C-n.m.r. data indicates the same orientation in the series of compounds **3**–**5**.

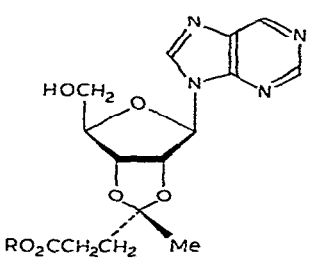
The acids **3b**, **4b**, and **5b** can be coupled with moist 6-aminohexylagarose (**6**) in 1:1 water–*p*-dioxane mediated by *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC). After 20 h, gels were isolated which contained 202, 126, and 176 μmol of bound ligand per g of the dry gel, respectively. For moist gels con-



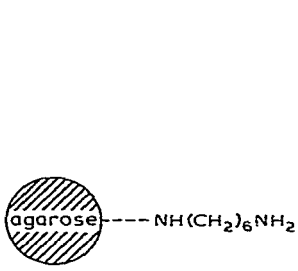
1 X = CH, R = NH_2
2a X = N, R = SH
2b X = N, R = H



3a X = CH, R = NH_2, R′ = Et
3b X = CH, R = NH_2, R′ = H
4a X = N, R = SH, R′ = Et
4b X = N, R = SH, R′ = H



5a R = Et
5b R = H



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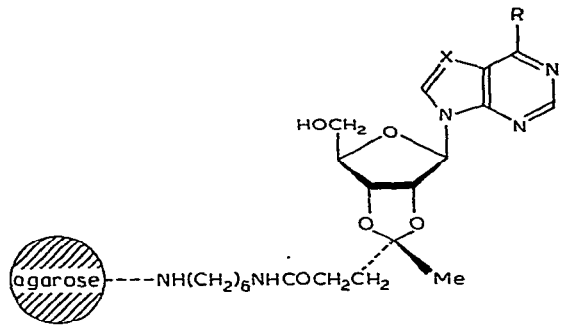

7a X = CH, R = NH_2
7b X = N, R = SH
7c X = N, R = H

taining 96% of water, these values become 8.08, 5.04, and 7.04 μmol/g for **7a**, **7b**, and **7c**, respectively.

Adenosine deaminase was retarded on gel **7a** (Fig. 1b) and was eluted with 0.15M sodium chloride. This result contrasts with the binding of 7-deazaadenosine, which does not inhibit deamination. A similar result was obtained with **3b**, which did not inhibit the adenosine deamination at an adenosine/**3b** ratio of 1 : 10. Therefore, the tubercidin ligand cannot be active site-directed and the affinity of the polymer **7a** can only be due to non-specific ionic or hydrophobic interactions. Binding forces can be characterised by minimum concentrations ($[Na^+Cl^-]_{min}$ in mmol) of aqueous sodium chloride necessary to elute the adsorbed enzyme. Below these minimum concentrations, the enzyme cannot be eluted within 35 void volumes. This "all-or-none" elution may be due to conformational changes in the enzyme, induced[17] by a so-called "deforming buffer". If the specific ratios of binding between adenosine deaminase and immobilised adenosine and inosine are compared, it can be shown that the enzyme is bound nearly five times more strongly to the substrate gel than to the product gel (Fig. 1c,d):

$$\frac{(Na^+Cl^-)^{A\text{-gel}}_{min} - (Na^+Cl^-)^{T\text{-gel}}_{min}}{(Na^+Cl^-)^{I\text{-gel}}_{min} - (Na^+Cl^-)^{T\text{-gel}}_{min}} = \frac{900 - 150}{300 - 150} = 5$$

If the K_m and K_i values for adenosine and inosine are compared[5] (Table I), it can be seen that the substrate is bound about five times more strongly to adenosine deaminase than is the product.

Chromatography of adenosine deaminase on the inhibitor gel **7c** (Fig. 1c) leads to a very strong interaction with the enzyme, and elution can only be accomplished with 1.4M NaCl. This finding agrees with the strong inhibition of adenosine deamination by nebularine and by the acid **5b** (Table II), and its low K_i value[14] (0.88×10^{-5}) at the monomer level. If the minimum salt concentration is calculated for the gel **7c** in a similar fashion to the substrate- and product-gel, it can be seen that there is no longer a numerical, but only a general, parallelism. Thus, immobilised inosine

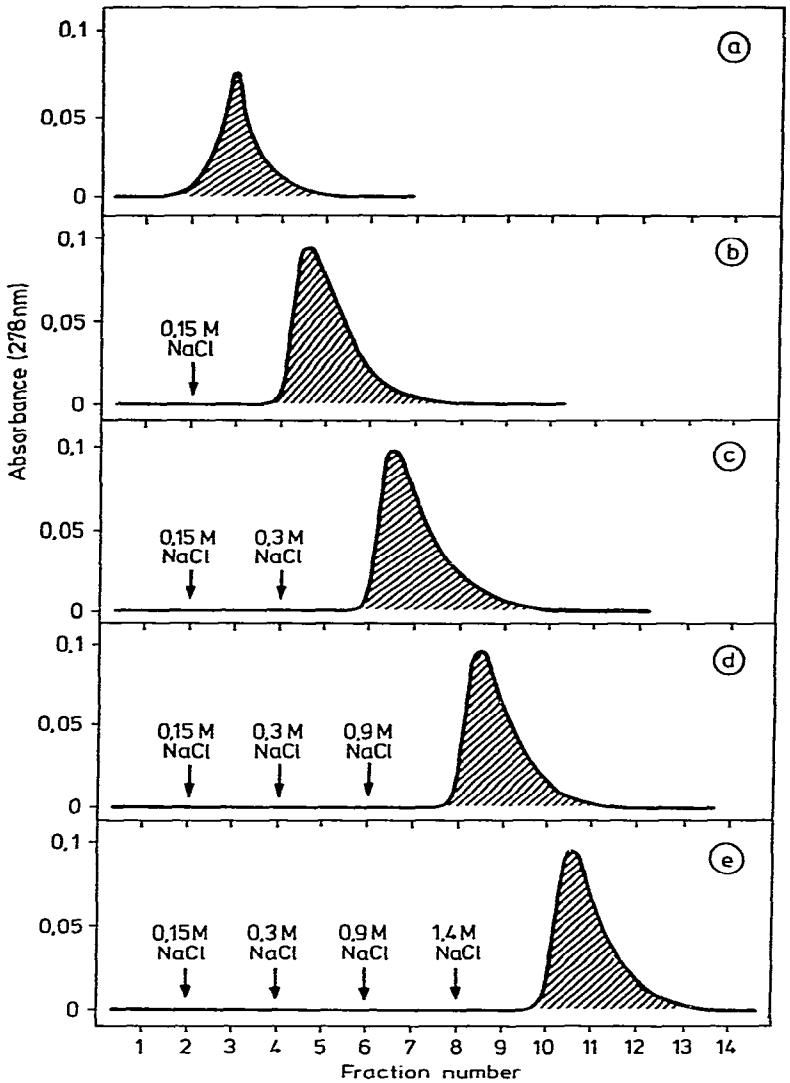

Fig. 1. Affinity chromatography of adenosine deaminase on the resins: (*a*) 6-aminohexylagarose, (*b*) 7a, (*c*) immobilised inosine, (*d*) immobilised adenosine, and (*e*) 7c.

TABLE I

MINIMUM CONCENTRATIONS OF NaCl FOR ELUTION OF ADENOSINE DEAMINASE FROM AFFINITY GELS, AND K_m/K_i VALUES OF THE NUCLEOSIDES FOR THIS ENZYME

Ligand of affinity gel	*Minimum concentration of NaCl (mmol)*	K_m/K_i *for the nucleosides*
Tubercidin	150	—
Inosine (product-gel)	300[2]	16×10^{-5}
Adenosine (substrate-gel)	900[2]	3.1×10^{-5}
Nebularine (inhibitor-gel)	1400	0.88×10^{-5}

TABLE II

RELATIVE INITIAL VELOCITIES OF THE DEAMINATION REACTION OF ADENOSINE BY ADENOSINE DEAMINASE IN THE PRESENCE OF VARIOUS INHIBITORS

Compound	*Molar ratio adenosine/inhibitor*	*Relative initial velocity*
Adenosine	—	100
2′,3′-*O*-[1-(2-Carboxyethyl)ethylidene]tubercidin (**3b**)	1:10	100
2′,3′-*O*-[1-(2-Carboxyethyl)ethylidene]-6-thioinosine (**4b**)	1:10	42
2′,3′-*O*-[1-(2-Carboxyethyl)ethylidene]inosine	1:10	71
2′,3′-*O*-[1-(2-Carboxyethyl)ethylidene]nebularine (**5b**)	1:1	55
Inosine	1:10	79
6-Thioinosine (**2a**)	1:8	47
Nebularine (**2b**)	1:1	34

and nebularine (**7c**) are useful affinity gels for the isolation of adenosine deaminase. Furthermore, unspecific binding of affinity resins can be suppressed when salt concentrations are chosen which are slightly below the minimum concentration.

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 measured for solutions in Me_2SO-d_6 (internal Me_4Si) with Bruker HX-60 or WP-270 spectrometers, and chemical shifts are reported in δ values. U.v. spectra were measured with Zeiss PMQ 3 or Shimadzu UV-200 spectrometers. Microanalyses were performed by Mikroanalytisches Labor Beller, Göttingen, Germany.

Column chromatography was performed on Dowex-1X8 (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*, methanol–water (1:4); and *B*, 0.25M LiCl. Thin-layer electrophoresis (t.l.e.) was performed on silica gel in a TLE Double Chamber (Desaga, Heidelberg, Germany) with 0.1M sodium citrate (pH 6.5). Affinity chromatography was performed in thermostatted, jacketed, Multichrom columns (30 × 0.9 cm, Serva-Technik, Heidelberg, Germany) attached to an LKB Uvicord 3 photometer (at 278 nm). 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, **6**), tubercidin (7-deazaadenosine), 6-thioinosine, and adenosine deaminase (EC 3.5.4.4) were commercial products. Nebularine was prepared according to the method of Fox *et al.*[7].

Relative initial velocities of the deamination of adenosine by adenosine deaminase in the presence of various inhibitors were determined by addition of 2.5×10^{-5} units of adenosine deaminase to 1 ml of 0.07M phosphate buffer (pH 7.6)

containing 0.1 μmol of adenosine and 0.1–1.0 μmol of the inhibitor. The decrease of adenosine at room temperature was followed at 262 nm within 7 min. The slope between the first and second minute was defined as the initial velocity, and arbitrarily taken as 100 for adenosine.

2′,3′-O-[*1*-(*2-Ethoxycarbonylethyl*)*ethylidene*]*tubercidin* (**3a**). — To a suspension of tubercidin (2 g, 7.51 mmol) in dry *N,N*-dimethylformamide (25 ml) were added ethyl levulinate (2.5 ml, 15 mmol) and triethyl orthoformate (1.85 ml, 11.2 mmol), and insoluble material was dissolved by adding a solution (2.4 ml) of 7M hydrogen chloride in dry *p*-dioxane. The mixture was kept for 24 h at room temperature and then poured into ether (300 ml). The upper layer was decanted, and the oily residue was washed twice with ether and then dissolved in chloroform with the addition of 2% aqueous sodium hydrogen carbonate. The organic layer was washed with water, dried, and concentrated to dryness to give **3a** (2.4 g, 81.6%), R_F 0.25 (solvent *B*), λ_{max}^{MeOH} 271 nm (ε 11600). ^{1}H-N.m.r. data: δ 8.60 (s, H-2), 7.35 (d, *J* 3.5 Hz, H-6), 7.05 (s, NH_2), 6.60 (d, *J* 3.5 Hz, H-5), 6.18 (d, *J* 3.0 Hz, H-1′), 5.25 (q, H-2′), 4.95 (q, H-3′), 4.20 (q, H-4′), 4.03 (m, ester CH_2), 3.50 (t, H-5′), 2.30 (m, aliphatic CH_2), 1.30 (s, acetal Me), and 1.25 (t, *J* 7.5 Hz, ester Me). ^{13}C-N.m.r. data: δ 14.004 (ester Me), 23.547 (acetal Me), 28.215 (acetal CH_2), 33.367 (CH_2-C=O), 59.854 (O-CH_2), 61.583 (C-5′), 81.016 (C-2′), 83.436 (C-3′), 85.165 (C-4′), 88.865 (C-1′), 100.034 (C-5), 102.904 (C-9), 113.969 (acetal C), 122.198 (C-6), 149.723 (C-8), 151.866 (C-2), 157.572 (C-4), and 172.544 p.p.m. (C=O).

Anal. Calc. for $C_{18}H_{25}N_4O_6$: C, 55.06; H, 6.12; N, 14.28. Found: C, 55.09; H, 6.16; N, 14.19.

2′,3′-O-[*1*-(*2-Carboxyethyl*)*ethylidene*]*tubercidin* (**3b**). — A solution of **3a** (1 g, 2.55 mmol) in 1 : 1 ethanol and M sodium hydroxide (60 ml) was kept for 30 min at room temperature and then neutralised (glass electrode) with Amberlite IR-120 (H^+) resin, filtered, and concentrated. The residue was dissolved in water and, after addition of a few drops of conc. ammonia, eluted from a column (20 × 1.5 cm) of Dowex-1 X8 (AcO^-) resin with a linear gradient of 0.5M acetic acid (500 ml) and water (500 ml). The fractions containing the main peak were concentrated, and the residue was dried in a desiccator over sodium hydroxide to give **3b** (850 mg, 92%) as white, amorphous material, R_F 0.6 (solvent *B*); t.l.e. (relative to tubercidin) +2.4; λ_{max}^{MeOH} 272 nm (ε 11850). ^{1}H-N.m.r. data: δ 8.05 (s, H-2), 7.33 (d, *J* 3.5 Hz, H-6), 7.05 (bs, NH_2), 6.60 (d, *J* 3.5 Hz, H-5), 6.15 (d, *J* 3.0 Hz, H-1′), 5.20 (q, H-2′), 4.90 (q, H-3′), 4.60 (bs, OH), 4.10 (m, H-4′), 3.50 (m, H-5′), 2.10 (aliphatic CH_2), and 1.30 (s, acetal Me).

Anal. Calc. for $C_{16}H_{21}N_4O_6$: C, 52.74; H, 5.53; N, 15.37. Found: C, 52.90; H, 5.68; N, 15.14.

2′,3′-O-[*1*-(*2-Ethoxycarbonylethyl*)*ethylidene*]-*6-thioinosine* (**4a**). — The synthesis of **4a** was carried out as described for **3a**, starting with 6-thioinosine (10 g, 24.4 mmol). The resulting, oily residue was dissolved in 1-butanol with the addition of 2% aqueous sodium hydrogen carbonate. The organic layer was washed with water and concentrated, and the residue was recrystallised from methanol to give

4a (6.71 g, 82%), as yellowish crystals, m.p. 248–252°, R_F 0.87 (solvent *A*), λ_{max}^{MeOH} 325 nm (ε 23610). ^{1}H-N.m.r. data: δ 8.45 (s, H-2), 8.10 (s, H-6), 6.15 (d, *J* 2.0 Hz, H-1'), 5.2 (m, H-2'), 4.95 (m, H-3'), 4.20 (m, H-4'), 4.0 (q, *J* 7.5 Hz, ester CH_2), 3.5 (m, H-5'), 2.3 (m, aliphatic CH_2), 1.35 (s, acetal Me), and 1.2 (s, ester Me). ^{13}C-N.m.r. data: 176.313 (C-6), 172.440 (C=O), 145.158 (C-2), 143.41 (C-4), 141.147 (C-8), 135.57 (C-5), 113.865 (acetal C), 89.799 (C-1'), 87.205 (C-4'), 84.024 (C-3'), 81.293 (C-2'), 61.445 (C-5'), 59.854 (O-CH_2), 33.609 (CH_2-C=O), 28.284 (acetal CH_2), 23.478 (acetal Me), and 14.000 (ester Me).

Anal. Calc. for $C_{17}H_{22}N_4O_6S$: C, 49.75; H, 5.40; N, 13.65; S, 7.81. Found: C, 49.78; H, 5.49; N, 13.70; S, 7.84.

2',3'-O-[1-(2-Carboxyethyl)ethylidene]-6-thioinosine (**4b**). — Compound **4a** (1 g, 2.44 mmol) was saponified, as described for **3b**, to give **4b** (760 mg, 83%) as colorless crystals, m.p. 217–219°, R_F 0.57 (solvent *B*), λ_{max}^{MeOH} 325 nm (ε 22360); t.l.e. (relative to 6-thioinosine) +1.6. ^{1}H-N.m.r. data: δ 8.35 (s, H-2), 8.25 (s, H-6), 6.1 (d, *J* 2 Hz, H-1'), 5.4 (m, H-2'), 4.95 (m, H-3'), 4.2 (m, H-4'), 3.55 (d, *J* 5.0 Hz, H-5'), 2.2 (m, aliphatic CH_2), and 1.3 (s, acetal Me). ^{13}C-N.m.r. data: 190.013 (C-4), 172.338 (C=O), 146.487 (C-2), 143.261 (C-8), 139.505 (C-6), 135.175 (C-9), 113.566 (acetal C), 89.262 (C-1'), 86.39 (C-4'), 82.855 (C-3'), 80.645 (C-2'), 60.98 (C-5'), 33.805 (CH_2-C=O), 28.944 (acetal CH_2), and 22.978 (acetal Me).

Anal. Calc. for $C_{15}H_{18}N_4O_6S$: C, 47.12; H, 4.74; N, 14.65; S, 8.38. Found: C, 47.27; H, 4.84; N, 14.63; S, 8.25.

2',3'-O-[1-(2-Ethoxycarbonylethyl)ethylidene]nebularine (**5a**). — To a solution of **4a** (3 g, 7.3 mmol) in 1-butanol was added Raney nickel/water (6:4, 5 g) suspended in water. The mixture was heated for 2 h at 90°, and then filtered. The catalyst was washed with hot water (200 ml). The organic layer was separated from the combined filtrate and washings, and concentrated, and ether was repeatedly evaporated from the residue to give light-yellow, amorphous **5a** (1.8 g, 65.2%), R_F 0.70 (chloroform–methanol, 2:8), λ_{max}^{MeOH} 262 nm. ^{1}H-N.m.r. data: δ 9.18 (s, H-2), 8.98 (s, H-8), 8.82 (s, H-6), 6.37 (d, *J* 2 Hz, H-1'), 5.50 (m, H-2'), 5.1 (m, H-3'), 4.37 (m, H-4'), 4.13 (q, CH_2-ester), 3.53 (H-5'), 2.35 (q, aliphatic CH_2), 1.38 (s, acetal Me), and 1.27 (s, ester Me). ^{13}C-N.m.r. data: δ 172.776 (C=O), 152.313 (C-4), 150.823 (C-2), 148.298 (C-6), 145.578 (C-8), 134.309 (C-5), 114.04 (acetal C), 90.015 (C-1'), 87.424 (C-4'), 83.992 (C-3'), 81.596 (C-2'), 61.715 (C-5'), 60.096 (O-CH_2), 33.675 (CH_2-C=O), 28.429 (acetal CH_2), 23.637 (acetal Me), and 14.117 (ester Me).

Anal. Calc. for $C_{17}H_{22}N_4O_6$: C, 53.96; H, 5.86; N, 14.81. Found: C, 54.23; H, 6.10; N, 14.61.

2',3'-O-[1-(2-Carboxyethyl)ethylidene]nebularine (**5b**). — To a solution of **4b** (1 g, 2.61 mmol) in hot water was added, in portions, Raney nickel/water (1.7 g) suspended in water. The suspension was heated for 2 h at 90° and then filtered, and the catalyst was washed with hot water (100 ml). The combined filtrates were concentrated to 3 ml and treated with a few drops of ethanol. A solution of the resulting, crude **5b** (780 mg) in water (10 ml) was applied to a column (1 × 25 cm) of Lewatit CP 3050 (H^+) resin and eluted with water, to give **5b** (360 mg, 39.4%), R_F 0.38

(solvent *B*); t.l.e. (relative to nebularine) +1.57; λ_{max}^{MeOH} 261 nm (ε 5500); i.r.:ν_{max} 1565, 1380 (COO^-), 1280 (C=O), and 885 cm^{-1} (OH). ^{1}H-N.m.r. data: δ 9.17 (s, H-2), 8.97 (s, H-8), 8.77 (s, H-6), 6.30 (d, *J* 2.5 Hz, H-1′), 5.53 (q, *J* 2.5 Hz, H-2′), 5.0 (q, *J* 2.5 Hz, H-3′), 4.24 (q, *J* 2.5 Hz, H-4′), 3.68 (bs, OH), 3.56 (t, *J* 5.5 Hz, H-5′), 2.24 (q, *J* 9 Hz, aliphatic CH_2), and 1.34 (s, acetal Me).

Anal. Calc. for $C_{15}H_{18}N_4O_6$: C, 51.42; H, 5.18; N, 15.99. Found: C, 51.33; H, 5.30; N, 15.91.

Coupling reactions of 6-aminohexylagarose (**6**). — To a solution of **3b** (54.6 mg, 0.15 mmol) in water (10 ml) was added agarose gel (**6**, 5 ml) at pH 6. The suspension was shaken for 10 min and *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (100 mg, 0.52 mmol) was added. Shaking was continued overnight, and the agarose derivative was collected, washed with 0.1M sodium hydrogen carbonate (250 ml), 0.5M sodium chloride (250 ml), and water, to give the coupled product **7a** (5 g).

Portions (0.5 g) of moist **7a** and agarose **6** were dried for 72 h at 95° in high vacuum to constant weight. The dry powders (9.5 mg, each) and **3b** (10.0 μmol) were hydrolysed with 0.5M hydrochloric acid (5 ml) for 30 min at 100°. The absorbances at 245 nm (1-cm path-length) were 0.698, 0.415, and 1.505. Thus, dry **7a** contains 202 μmol of ligand/g.

The corresponding absorbances (at 325 nm) for dry **7b** and **6** (9 mg each) and **4b** (10 μmol), obtained as described above, were 0.505, 0.38, and 1.100, respectively. Thus, **7b** contains 126 μmol of ligand/g.

Likewise, the absorbances determined for **7c**, **6**, and **5b** were 0.725, 0.585, and 0.886, respectively. Thus **7c** contains 158 μmol of ligand/g.

Affinity chromatography of adenosine deaminase on **7a** *and* **7c**. — The gels **7a** and **7c** were thoroughly washed with water, packed in columns (20 × 0.9 cm), and equilibrated with twice the void volume of 0.07M phosphate buffer (pH 7.6). For each chromatographic run at 25°, 0.1 A_{278} unit of adenosine deaminase was used. Stepwise elution (0.05M NaCl, 2-ml fraction) was monitored on the basis of absorbance at 278 nm, and was quantitative. For minimum concentrations, see Table I.

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