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The Synthesis of Haptens and Properties of Catalytic Antibodies Designed to Catalyse Carbanionic Cyclisation Reactions

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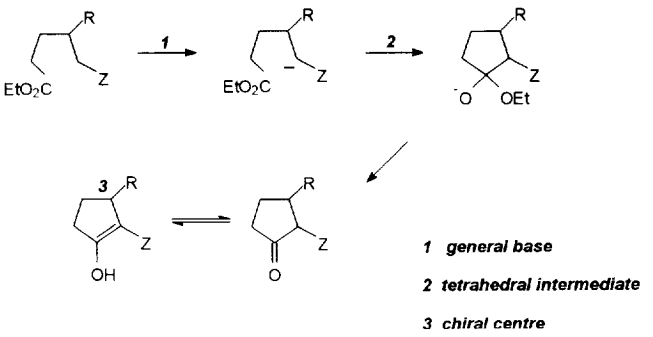
Abstract The design of haptens to generate antibodies with the potential to catalyse carbanionic cyclisation reactions is introduced in the context of Dieckmann cyclisations and related reactions. Syntheses of five-membered ring haptens containing a sulphone, to represent the transition state(s) associated with the tetrahedral intermediate, and an amino group, to promote the formation of a general base, are described. Results with partially purified antibodies are reported showing that the target reaction can be catalysed by antibodies.

Catalytic antibodies have now been described for a wide range of organic reactions[1]. The majority of these reactions have been promoted by haptens that have been designed to stabilise one transition state of a reaction profile. In a few cases, the hapten has been 'bifunctional', that is chosen to provide two catalytic components to the reaction as in the so-called 'bait and switch' strategy for hydrolytic antibodies[2]. In most cases also, the chief interest has been to demonstrate catalysis or to study mechanism and applications of catalytic antibodies have not been extensively described. Examples of a potential therapeutic antibody[3] and of an antibody with potential in preparative chemistry[4] have been described. We have been interested in catalysing reactions of value for the preparation of building blocks for synthesis[5]. The formation of carbon-carbon bonds is crucial in synthetic organic chemistry and in particular, reactions involving carbanionic intermediates play a major role. The Dieckmann reaction is a well established and well studied example[6] and is suitable for a pilot investigation in catalytic antibodies because it is a unimolecular cyclisation. This reaction was therefore selected for study.

In order to catalyse a Dieckmann-type cyclisation, an antibody must be able to accomplish at least two key steps (scheme 1). Firstly, a stabilising group such as alkoxycarbonyl or cyano. Secondly, the formation of the cyclic transition state must be encouraged. With the needs of organic synthesis in mind, it is also of interest to include a substituent to afford chiral centre. In order to avoid complexities associated with multiple conformations, we concentrated upon five-membered rings. There is significant evidence in the literature that the presence of an ammonium group in a hapten will result in a complementary carboxylate in the antibody, a group that is capable of promoting general base catalysis[7,8]; such a group was therefore included in the hapten structure. The stabilisation of tetrahedral transition states in acyl transfer reactions has generally been achieved using phosphonates or phosphonamidates. Since tetrahedral sulphur(VI) derivatives are known to be inhibitors of enzymes that catalyse acyl transfer reactions,[9] it is also possible that tetrahedral sulphur derivatives would also be applicable to catalytic

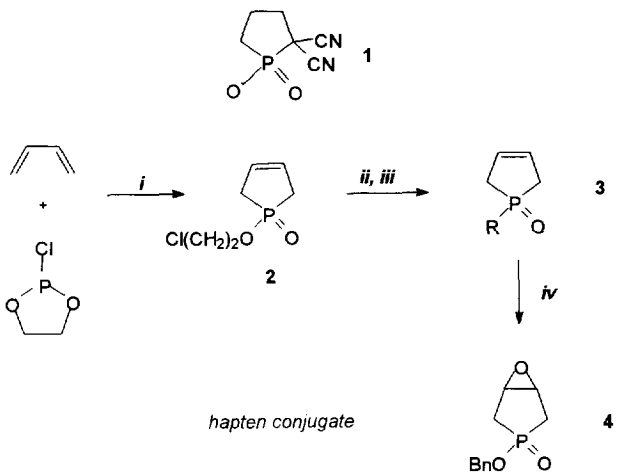
antibody chemistry.



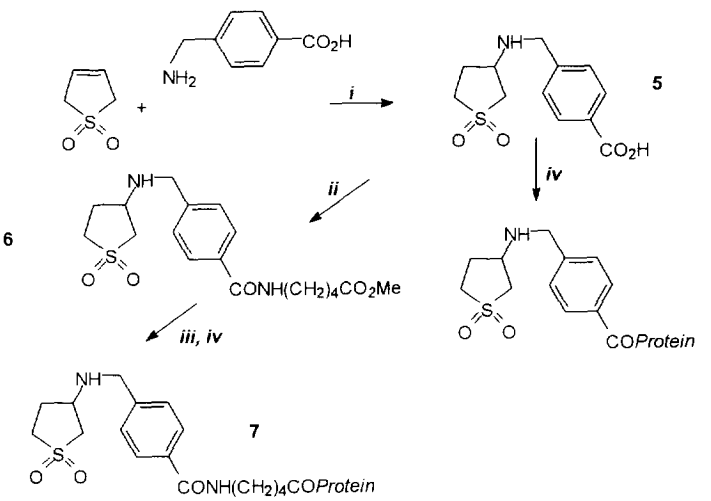
Scheme 1. Requirements for catalysis of cyclisation with carbanion intermediate.

Synthesis of Haptens. In order to minimise the catalytic power required by an antibody to promote a carbanionic cyclisation, the first hapten structures that we attempted to synthesise contained an α-dinitrile (**1**); the substrate complementary to such a hapten would be readily ionised at near neutral pH. Several routes were investigated involving carbanionic or electrocyclic reactions but, although some potentially useful intermediates were obtained, the synthesis was not concluded. In many applications of catalytic antibodies, it is now becoming clear that it is advisable to synthesise the simplest possible relevant hapten and to rely upon sensitive screening to identify the antibody required. We therefore removed the electron withdrawing substituent from the hapten structure and concentrated upon the two groups identified above as essential for the catalytic activity of interest. Both phospholanic acid derivatives and sulphones were investigated. The former were approached (scheme 2) through electrocyclic addition of 2-chloro-1,3-dioxaphospholane and butadiene[10] giving the 2,5-dihydrophosphole chloroethylester **2** which was converted into 1-chloro-2,5-dihydrophosphole-1-oxide using phosphorus pentachloride[10]. To assist in the manipulation of the small molecules, the benzyl ester **3** was prepared following Benkovic[11] and the principal hapten intermediate **4** was obtained by epoxidation with 3-chloroperbenzoic acid. The epoxide **4** could be conjugated directly to a basic immunogenic protein or coupled with a linker molecule prior to conjugation. Because useful haptens were obtained with sulphones and antibodies raised, no immunology was carried out with the phospholanic acid hapten derivatives. The sulphone haptens (scheme 3) were prepared by modifications of established methods[12]. Thus 3-sulpholene was reacted with 4-(aminomethyl)benzoic acid in the presence of potassium hydroxide to give the aminosulphone carboxylic acid **5**. This compound obtained in one step has all of the requirements for antibody generation and was indeed used for conjugation. However it is more usual to separate the hapten specifying the catalytic reaction from the immunogenic protein by means of a short linking molecule. The hapten **5** was therefore extended by coupling with methyl 4-aminobutanoate using isobutyl chloroformate in the presence of N-methylmorpholine to give **6**; hydrolysis of the methyl ester with sodium hydroxide afforded the extended hapten **7**.

The two sulphone haptens were separately coupled with bovine serum albumin and human transferrin to provide the materials necessary respectively for immunisation and identification of hapten-binding antibodies by ELISA.



Scheme 2. Synthesis of phospholanates. *Reagents*: i, Δ; ii PCl_5; iii, $PhCH_2OH$, Et_3N; iv, *m*-chloroperbenzoic acid

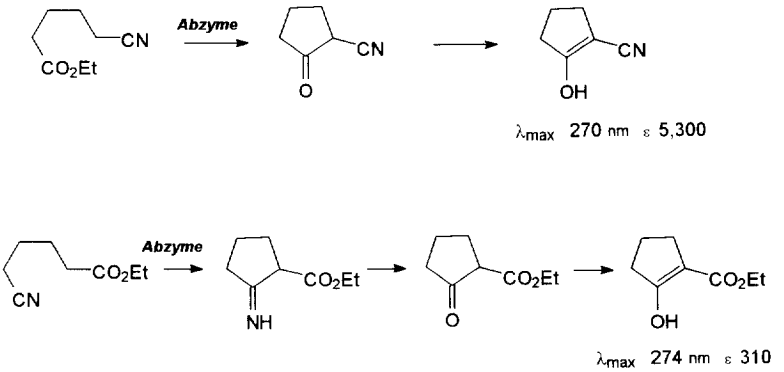


Scheme 3. Synthesis of sulphone haptens. *Reagents*: i, KOH; ii, *i*BuOCOCl, N-methylmorpholine; iii, aq. NaOH; iv, EDC, N-hydroxysuccinimide.

Preparation of antibodies. Antibodies were prepared by immunisation of NZB/Balb-c F1 hybrid female mice with conjugates of the sulphone haptens with bovine serum albumin (7-18 mol. hapten: 1 mol BSA) over a period of 4 months (see experimental section). Spleen cells from animals with a significant anti-hapten antibody titre were fused with myeloma cells (X63.Ag8.653) and hybridoma cells produced by standard procedures[13]. Assays for catalysis were carried out using concentrated supernatant solutions.

Evaluation of cell lines produced. Antibodies raised to the sulphone haptens have been obtained and shown to

possess some of the properties expected of a catalyst of Dieckmann-type cyclisations using ethyl 5-cyanopentanoate and diethyl hexandioic acid as substrates by uv, hplc, and glc assays[14]. The uv assay depends upon the absorption of the enol form of the cyclised products at 270-275 nm, the intensity of which is particularly favourable for 2-cyanocyclopentanone (scheme 4).



Scheme 4. Reactions for evaluation of monoclonal antibody cell-lines.

One cell line (2C9.D9) consistently showed a substantial increase in absorption at this wavelength when incubated with the substrate 5-cycanopentanoate whereas control experiments using reaction medium and other antibodies, including those derived from structurally unrelated haptens, showed no such increase (figure 1). That this increase in absorbance was due to cyclopentanones was confirmed by both glc and hplc assays. Both possible reaction products, 2-cyanocyclopentanone and 2-ethoxycarbonylcyclopentanone were detected in those reactions in which the uv absorbance increase had been observed (figure 2). The conversions obtained in several repeat experiments were substantial being in the range 10 - 45% of initial substrate into the total products over a period of 24 - 52 hours. No trace of product was found in control reactions using any of the three assays. Unfortunately, the cell line 2C9.D9 proved unstable in its ability to produce the catalytic antibody and it was therefore impossible to characterise its kinetic properties further with the available preparations. We are undertaking further experiments to obtain further active samples. Nevertheless, the evidence presented in this paper indicates that antibody catalysis of a complex multi-step reaction such as cyclisation via carbanion intermediates is possible.

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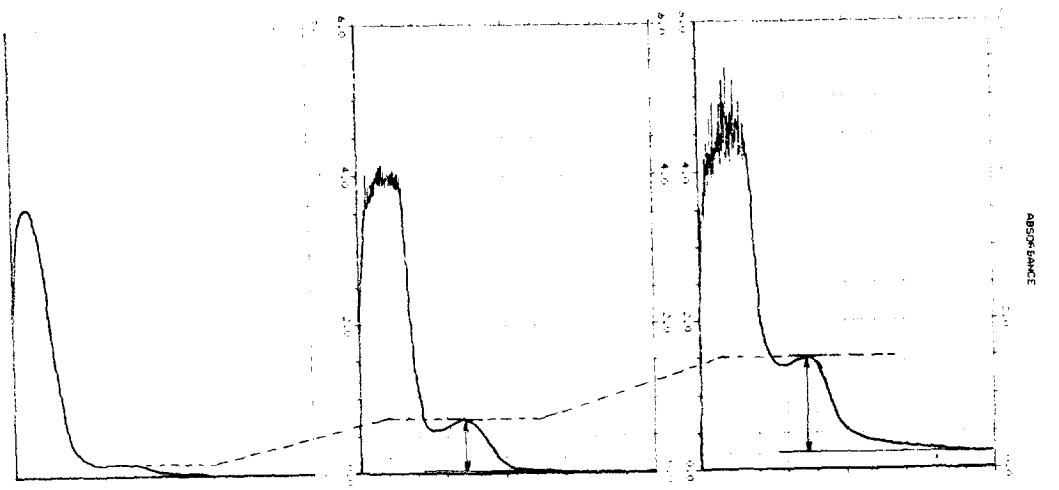
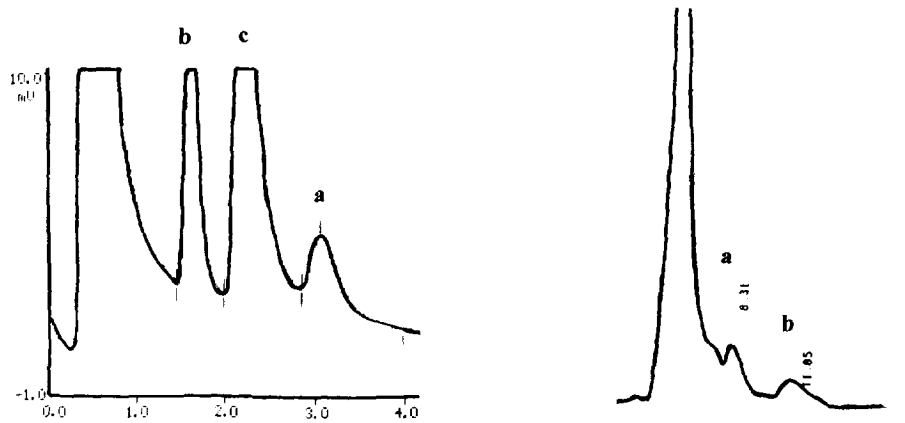
Experimental

^{1}H NMR spectra were recorded on a Bruker instrument operating at 400 and 250 MHz. ^{13}C NMR spectra were recorded on Bruker instruments operating at 100 and 63 MHz and were ^{1}H decoupled. ^{31}P NMR spectra were recorded on a Bruker instrument operating at 162 MHz and were ^{1}H decoupled. Except where otherwise stated, the solvent was $CDCl_3$. UV spectra were recorded on a Perkin-Elmer Lambda 2 instrument. FTIR spectra were recorded on a Unicam Mattson 1000 instrument as liquid film unless otherwise stated. High resolution Mass Spectra were run on a MS 902 instrument using Electron Impact ionisation.



(a) (b) (c)

Figure 1. Uv assay of cell line 2C9.D9 using ethyl 5-cyanopentanoate as substrate. The absorbance marked is at 276 nm. (a) non-catalytic antibody from an unrelated cell-line, (b) catalytic antibody after 0.5 h incubation, (c) catalytic antibody after 52h incubation.



(a) (b)

Figure 2. Hplc and glc assays of reaction mixtures from cell line 2C9.D9 using ethyl 5-cyanopentanoate as substrate. (a) Hplc (C18 reverse phase) (b) Glc (FFAP). The peaks marked correspond to **a** 2-cyanocyclopentanone, **b** 2-ethoxycarbonylcyclopentanone, and **c** ethyl 5-cyanopentanoate.

1-(2-Chloroethoxy)-2,5-dihydrophosphole-1-oxide[10] **(2)** Butadiene (10 ml; 0.11 mole), 2-chloro-3,2-dioxaphospholane (10 ml; 0.11 mole) and 2,6-di-*tert*-butyl-4-methylphenol (200 mg) was evenly split between two sealed stainless steel reaction vessels which were immersed in an oil bath at 100°C for 6 h. After standing at room temperature for 3 days the excess chlorophosphite was destroyed by careful addition of water. The aqueous phase was continuously extracted with chloroform for 48 h. The chloroform extracts were separated, dried (sodium sulphate), filtered and evaporated to give yellow liquid which was distilled (Kugelrohr) to give the title compound as a colourless liquid 8.97 g; 89%. B.p. 150°C (0.1 mm) [Lit.[10] b.p. 154-155°C (10 mm)]. (Found: m/z 180.0103 $C_6H_{10}ClO_2P$ requires M$^+$ m/z 180.0107). ν_{max}/cm^{-1} 1610 (C=C), 1250 (P=O). δ_H [400 MHz] 5.82 (2H, d, $^3J_{PH}$ 35.6 Hz, H-3,4), 4.20-4.15 (2H, m, H-α), 3.62 (2H, t, J_{HH} 5.6 Hz, H-β), 2.39-2.37 (4H, m, H-2.5). δ_C [100 MHz] 126.8 (d, $^2J_{PC}$ 16.1 Hz, C-3,4), 64.34 (d, $^2J_{PC}$ 6Hz, C-α), 43.06 (d, $^3J_{PC}$ 7Hz, C-β), 29.27 (d, $^1J_{PC}$ 90.6 Hz, C-2.5). δ_P 77.46.

1-Chloro-2,5-dihydrophosphole-1-oxide Phosphorus pentachloride (6.92g, 33.2 mmol) was added to the ester **2** (6g, 33.2 mmol) carefully (caution: exotherm). The mixture was then heated with stirring under nitrogen on a steam bath for s h. The required product was distilled from the reaction mixture underreduced pressure b.p. 116-118°C (24 mm). [Lit.[13,15] 63-65°C (0.1 mm)]. δ_H [250 MHz] 5.99 (2H, d, $^3J_{PH}$ 38.7 Hz, H-3,4), 2.93-2.87 (4H, m, H-2.5).

1-Benzyloxy-2,5-dihydrophosphole-1-oxide[11] **(3)** 1-Chloro-2,5-dihydrophosphole-1-oxide (2.71 g 19.8 mmol) was dissolved in dry dichloromethane (25 ml) and stirred under nitrogen at O°C. To this was added freshly distilled triethylamine (2g 19.8 mmol) in dichloromethane (5 ml). After stirring for 10 min., benzyl alcohol (2.14 g, 19.8 mmol) in dichloromethane (5 ml) was added. The reaction mixture was left stirring overnight and allowed to warm to room temperature. The mixture was evaporated to dryness and the residue redissolved in dichloromethane and white insoluble material filtered off. The solution was evaporated to dryness and the residue treated with ether giving a further solution and white insoluble material which was filtered off. The filtrate was evaporated to dryness affording an oily residue (4.38 g) which was purified by distillation (Kugelrohr) b.p. 110°C (1.5 mm). The title compound was obtained as a colourless oil (3.78g, 92%). (Found: m/z 208.0641 $C_{11}H_{13}O_2P$ requires M$^+$ m/z 208.0653). ν_{max}/cm^{-1} 1625 (C=C), 1268 (P=O), δ_H [400 MHz] 7.34-7.24 (5H, m, ArH), 5.82 (2H, d, $^3J_{PH}$ 33.3 Hz, H-3,4), 5.03 (2H, d, $^3J_{PH}$ 8.9 Hz, OCH_2), 2.41-2.24 (4H, m, H-2,5). δ_C [100 MHz] 136.22 (d, ArC), 128.58 (d, ArCH), 128.25 (d, C-3,4), 127.95 (d, ArCH), 126.89 (d, ArCH), 66.26 (d, OCH_2), 29.37 (d C-2.5), δ_P 76.01.

1-Benzyloxy-3,4-epoxy2,3,4,5-tetrahydrophosphole-1-oxide **(4)**[16] Benzyl ester **8** (3.78 g; 18.17 mmole) and *meta*-chloroperbenzoic acid (3.45 g; 19.99 mmole) were dissolved in dichloromethane (30 ml) and heated together at reflux for 3 days. The reaction mixture was allowed to cool and left to stand at room temperature for 3 days. The CH_2Cl_2 solution was washed with saturated sodium bicarbonate (20 ml), 5% aqueous sodium thiosulphate (15 ml), water (20 ml) and brine (15 ml). The CH_2Cl_2 solution was then dried (sodium sulphate), filtered and evaporated to give a colourless liquid 2.95 g; 72%. b.p.172-174°C (0.5 mm). (Found: m/z 224.0601 $C_{11}H_{13}O_3P$ requires M$^+$ m/z 224.0602). ν_{max}/cm^{-1} 1268 (P=O). δ_H [400 MHz] 7./39-7-31 (5H, m, ArH), 5.05-5.01 (2H, m, OCH_2), 3.60-3.49 (2H, dm (X2), H-3,4), 2.17-2.03 (4H, m, H-2.5). δ_C [100 MHz] 136 (ArC), 128.85-127.91 (ArCH), 66.8-66.5 (OCH_2), 53.2-52.7 (C-3.4), 28.9-27.1 (C-2.5). δ_P 77.25, 73.93.

4-(N-(3-Tetrahydrothiophenyl-1,1-dioxide)aminomethyl)benzoic acid **(5)** 3-Sulpholene (6 g; 69.77 mmole), 4-(aminomethyl)benzoic acid (10.96 g; 72.70 mmole) and potassium hydroxide (6 g) were mixed together in water (100 ml). The mixture was then heated with stirring in an oil bath at *ca.* 55°C for 23 h. Tlc (SiO_2; 50% EtOAc/n-

hexane) showed no 3-sulpholene remainded. The pH of the mixture was adjusted to pH7 with conc. HCl and the volume reduced by about half. A white solid precipitates and this was filtered off while the solution was still hot, as the solution cooled more solid precipitates, once cold this solid was then also filtered off. The filtrate was shown to contain mainly the title compound by hplc analysis and this was further purified by flash column chromatography (20% MeOH/CH_2Cl_2 with 1% Et_3N added) to give the required compound as a white solid which was washed with hot water and dried under vacuum over P_2O_5. Yield (95%) m.p. 224-6°C (decomp.) (Found: 53.04% C, 5.61% H, 5.08% N, $C_{12}H_{16}NO_4S$ requires 53.52% C, 5.61% H, 5.20% N, m/z 269.1343 $C_{12}H_{16}NO_4S$ requires 269.0724). ν_{max}/cm^{-1} (KCl) 1595, 1550 (CO_2-), 1310, 1115 (SO_2). λ_{max}/nm (MeOH) 247.9 ε_{max} 966. δ_H [400 MHz, NaOD/D_2O] 8.04 (2H, d, J 8.2 Hz, ArH), 7.53 (2H, d, J 8.1 Hz, ArH), 3.91 (2H, s, OCH_2), 3.72-3.68 (H, m, H-3), 3.64-3.59 (H, m, H-2a), 3.55-3.49 (H, m, H-2b), 3.35-3.27 (H, m, H-5a), 3.18-3.13 (H, m, H-5b), 2.67-2.59 (H, M, H-4a), 2.24-2.14 (H, m, H-4b). δ_C [100 MHz, NaOD/D_2O] 175.08 (C, C=O), 141.6 (C, ArC), 135.69 (C, ArC), 129.27 (CH, ArCH X2), 128.33 (CH, ArCH X2), 55.65 (CH_2, OCH_2), 53.54 (CH, C-3), 50.84 (CH_2, C-1), 50.35 (CH_2, C-5), 28.55 (CH_2, C-4).

N-(3-Carbomethoxypropyl)-4-(N'-(3-tetrahydrothiophenyl-1,1-dioxide)aminomethyl)benzamide (6) Sulphone **5** (0.217 g; 0.807 mmole) was suspended in a mixture of DMF (10 ml) and THF (25 ml) and N-methylmorpholine (0.4 ml; 3.64 mmole) added. This suspension was cooled in an ice bath and isobutyl chloroformate (0.2 ml; 1.54 mmole) was added with stirring. The reaction mixture was stirred at this temperature for 20 min. then mthyl 4-aminobutyrate hydrochloride (0.305 g; 1.99 mmole) was added in one portion. The reaction was allowed to warm slowly to room temperature and left to stir overnight. The reaction mixture was evaporated to dryness and the resulting clear oil was taken up in hot ethanol and left to stand overnight. The title compound was filtered off as a white solid (0.18 g; 61%) m.p. 183-5°C. (Found: m/z 368.1408; $C_{17}H_{24}N_2O_5S$ requires 368.1406) δ_H [250 MHz, D_2O] 7.77 (2H, d, J 8.1 Hz, ArCH), 7.57 (2H, d, J 8.1 Hz, ArCH), 4.37 (2H, d, J 4.5 Hz, benzylic NCH_2) 4.43-4.21 (H, m, H-3), 3.83-3.74 (H, m, H-2a), 3.59 (3H, s, OCH_3), 3.54-3.49 (H, m, H-2b), 3.42 (2H, t, J 6.4 Hz, NCH_2), 3.34-3.27 (2H, m, H-5), 2.9-2.8 (H, m, H-4a), 2.47-2.3 (3H, m, H-4b and CH_2CO_2Me), 1.95-1.84 (2H, m, CH_2). δ_C [63 MHz] 176.99 (C=O, ester), 170.22 (C=O, amide), 135.35 (ArC), 134.11 (ArC), 130.51 (ArCH X2), 128.33 (ArCH X2), 52.87 (NCH_2, benzylic), 52.55 (C-3), 51.79 (OCH_3), 50.25 (C-2), 49.83 (C-5), 39.57 (NCH_2), 31.58 (CH_2CO_2Me), 25.81 (C-4), 24.21 (CH_2).

N-(3-carboxylpropyl)-4-(N'-(3-tetrahydrothiophenyl-1,1-dioxide)aminomethyl)benzamide (7) The methyl ester **6** (44 mg; 0.12 mmole) was dissolved in water (0.5 ml) and sodium hydroxude (200 mg) added and the mixture left to stand for 15 min. before evaporating to dryness. The white solid residue was taken up in the minimum amount of hot water and acidified with conc. HCl. The solvent was again evaporated and the solid residue dissolved in hot water. Once cooled the resulting white solid was filtered off and dried under vacuum over P_2O_5 m.p. 155-157°C. (Found: 54.37% C, 6.41% H, 7.47% N, $C_{16}H_{20}N_2O_5S$ requires 54.22% C, 6.41% H, 7.90% N, m/z 352.1115 $[M-2H]^+$. $C_{16}H_{20}N_2O_5S$ requires M' m/z 352.1093). λ_{max}/nm (H_2O) 256.1, ε_{max} 634. δ_H [400 MHz, d_5-pyridine] 9.24 (H, bm), 8.70 (2H, bs, under pyridine peak), 8.24 (2H, d, J 8.1 Hz, ArH), 7.48 (2H, d, J 8.0 Hz, ArH), 3.82-3.76 (4H, m, 2 x NCH_2), 3.64 (H, quin, J 6.3 Hz, H-3), 3.54-3.49 (H, m, H-2a), 3.45-3.38 (H, m, H-2b), 3.24-3.11 (2H, m, H-5), 2.66 (2H, t, J 7.4 Hz, CH_2CO_2-), 2.41-2.35 (H, m, H-4a), 2.24 (2H, t, J 7.1 Hz, CH_2), 2.19-2.14 (H, m, H-4b). δ_C [63 MHz, NaOD/D_2O] 183.11 (C=O, acid), 179.00 (C=O, amide), 142.77 (ArC), 132.70 (ArC), 129.47 (ArCH x2), 127.68 (ArCH x2), 53.50 (NCH, benzylic) 51.19 (C-3), 50.41 (C-25), 40.07 (NCH_2), 35.31 (CH_2CO_2H), 28.64 (C-4), 25.79 (-CH_2-).

Preparation of antibodies. Haptens **5** and **6** were coupled to bovine serum albumin to give a ration of 7-18

mol.equiv. hapten: 1 BSA. The conjugates were employed to immunise NZB/Balb-c F1 hybrid female mice (7-9 weeks) over a 4 month period. Injections (150μg protein / 0.2 ml 0.15M NaCl) were given intraperitoneally every 2 weeks, initially with an equal volume of Freund's Complete Adjuvant and thereafter with Freund's Incomplete Adjuvant. Four days before sacrifice, animals received a single intravenous injection of 200 μg protein in 0.1 ml 0.15m NaCl. Spleen cells were obtained only from mice that had peripheral blood anti-sulphone antibody titres greater than 1 in 10^4. These were fused with myeloma cells (X63.Ag8.653) as reported previously[16] and hybridoma cells obtained by standard procedures. Microtitre plate wells containing hybrids secreting antibody to the hapten immunogen were identified by ELISA using the identical sulphone conjugated to human transferrin as the plate coating. Thereafter, hybridomas were only expanded and cloned if the antibody being secreted was subject ot greater than 90% competition in its binding in the ELISA system in the presence of 1μg / ml unconjugated hapten. Seven fusions were carried out with lymphocytes derived from mice immunised with **7** and five mice immunised with **5**. Specific antibody competing as described in the previous paragraph was produced in 36 and 23 microtitre plate wells using haptens **7** and **5** respectively. However only two showed catalytic activity as demonstrated in figure 1; these were designated 3F7.C9 and 2C9.D9, both of which were derived from hapten **7**. The cell lines were grown in 150ml culture flasks (Falcon) in RPMI 1640 medium containing 5% v/v foetal calf serum and 50μg / ml gentamycin. The supernatants were harvested and the immunoglobulin concentrated by precipitation with 50% saturated aqueous ammonium sulphate solution. For assay, the antibody was redissolved in 0.02M sodium phosphate buffer, pH 7.4 or pH 8, containing 0.15M NaCl (PBS).

Assay of monoclonal antibody preparations. Reactions mixtures were composed of PBS (pH 8.0, 4 ml), acetonitrile (1ml), antibody solution (200 μl), and substrate (20 mg) and were run at room temperature. Samples were removed for analysis by uv spectrosopy and chromatography. Glc analyses were carried out on FFAP (injector 240°C, oven 220°C, detector 270°C). Hplc assays were carried out using octadecylsilyl reverse phase columns (Sperisorb ODS2 or Deltapak C18) eluting with 55% acetonitrile/water, flow rate of 1 ml min^{-1}, detection by uv absorption (220 nm).

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