Remarkably Efficient Hydrolysis of a 4-Nitrophenyl Ester by a Catalytic Antibody Raised to an Ammonium Hapten

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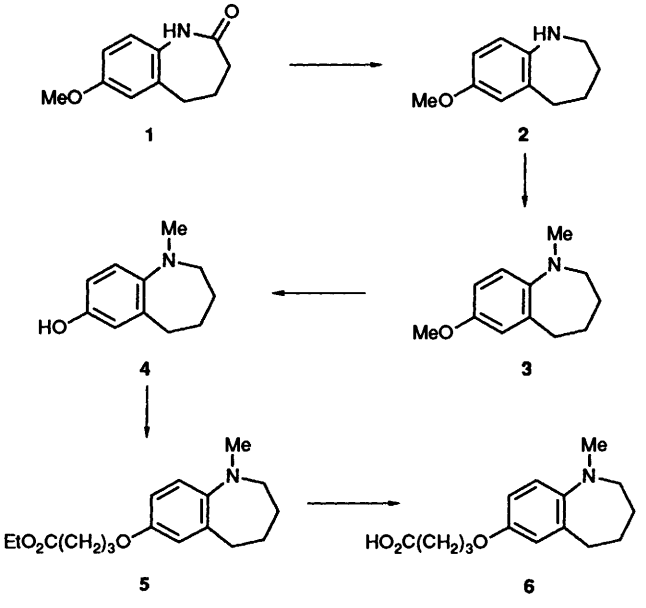
Antibodies have been raised to a 1-benzazepine hapten and the properties of two of the strongly binding clones, designated C3 and C5, as catalysts examined. Neither antibody catalysed the reaction for which they were first generated, electrophilic substitution in the benzene ring, but C3 catalysed the hydrolysis of an aralkyl 4-nitrophenyl ester with a rate enhancement of more than 10^6 compared with the background solvolysis rate. The mechanism of the hydrolysis reaction is suggested to involve general base catalysis on the basis of chemical modification experiments and isotope effects on the reaction rate in D_2O. The possibility that C3 might catalyse other reactions (elimination, deuterium exchange, and epoxide opening) was investigated but no other reactions were observed. In contrast, C5 catalysed none of the reactions investigated. The properties of the two antibodies are discussed with respect to their ability to bind compounds structurally related to the hapten.

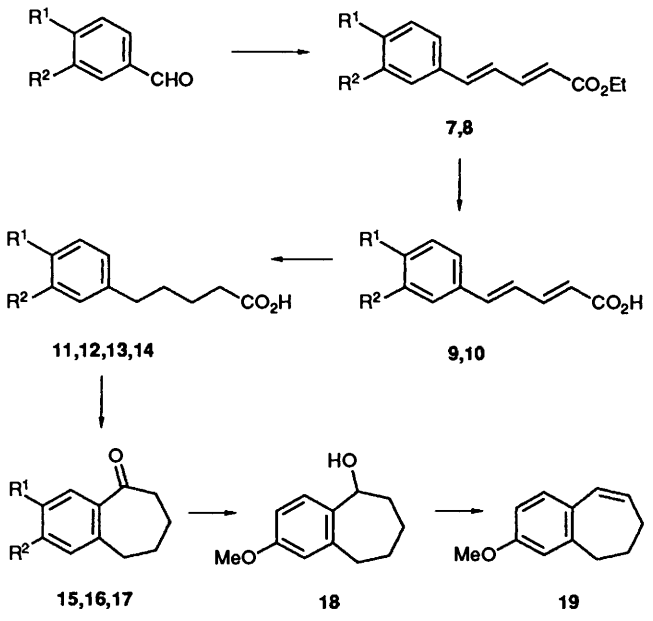
Catalytic antibodies (abzymes) have been reported to catalyse many hydrolyses[1] but the catalysis of reactions of potential synthetic interest has received relatively little attention, the most notable example of success being the Diels–Alder reaction.[2–4] The guiding principle for designing haptens to which antibodies are raised has been the identification of transition state analogues for the reaction in question.[1] However, in a small number of cases, attention has been paid to generating functional groups that may take part in catalysis through the recognition of a complementary functional group in the hapten; thus ammonium compounds as haptens have been shown to be bound by antibodies that contain carboxylate groups which act as general bases.[5,6] We were interested in the possibility that abzymes might contribute to catalysis of electrophilic aromatic substitution, a reaction for which only a few specialised enzymes such as prenyltransferases, thymidylate synthetase, cosynthetase (tetrapyrroles) and tryptophan synthetase are known. Aromatic substitution is well understood to proceed through cationic intermediates. Accordingly, as a first step, we investigated the properties of antibodies raised to a hapten **6** the protonated form of which can be compared with positively charged intermediates in substitution.



Scheme 1

Synthesis of Hapten and Related Compounds.—The synthetic routes to the hapten followed established procedures[7,8] and are shown in Scheme 1. Thus 6-methoxy-α-tetralone was converted into its oxime (92%) which was converted into the tosylate prior to Beckmann rearrangement with dilute acetic acid to give the known lactam **1** (50%). Reduction of the lactam with lithium aluminium hydride afforded the tetrahydrobenzazepine **2** (94%)[7] which was *N*-methylated with iodomethane (94%).[8] The *O*-methyl group was then cleaved with boron tribromide to give the phenol **4** (87%) and the linker for attachment of the hapten to the immunogenic protein was introduced by alkylation with ethyl 4-bromobutanoate giving **5** (83%). The ethyl ester was hydrolysed (76%) and the active ester for coupling prepared by reaction of the acid with *N*-hydroxysuccinimide and carbodiimide. Conjugates **I** and **II** were then prepared with bovine serum albumin (BSA) and human transferrin (Tf). These conjugates were then used to obtain the antibodies as described previously.[9]

In order to evaluate the properties of the antibodies, we required also several related compounds the preparation of which is summarised in Scheme 2. 5-(3-Methoxyphenyl)- **11** and 5-(3-hydroxyphenyl)-pentanoic acid **12** were prepared from 3-methoxybenzaldehyde by condensation with ethyl crotonate to give ethyl 5-(3-methoxyphenyl)penta-2,4-dienoate **7** which was successively hydrolysed and hydrogenated to afford **11**.[10] Demethylation was accomplished with boron tribromide as before to give the phenol **12**. The isomeric 4-hydroxyphenylpentanoate **14** was prepared following a parallel sequence from 4-methoxybenzaldehyde. The cyclisation product **15** of the 3-methoxy series was obtained by treatment of the acid **11** with polyphosphoric acid. The 3-hydroxybenzocycloheptenone **17** was obtained from benzosuberone in three steps *via* the corresponding nitro and amino compounds.[11] In order to investigate the ability of C3 and C5 to catalyse cyclisation reactions, the ethyl, ethylthio, 4-chlorophenyl, and 4-nitrophenyl esters **20**–**23** of 5-(3-methoxyphenyl)pentanoic acid were prepared. Further compounds prepared to investigate other



	7	9	11	12	8	14
R^1	H	H	H	H	OMe	OMe
R^2	OMe	OMe	OMe	OH	H	H

	13	14	15	16	17
R^1	OMe	OH	H	H	OH
R^2	H	H	OMe	OH	H

Scheme 2

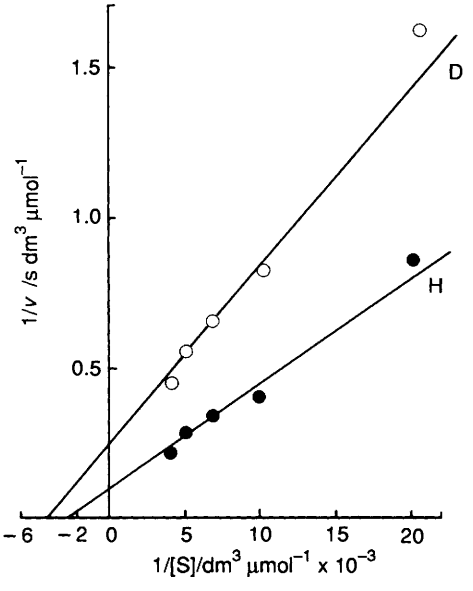
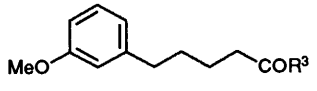


Fig. 1 Michaelis–Menten plot of the hydrolysis of **23** catalysed by antibody C3



20 R^3 = OEt
21 R^3 = SEt
22 R^3 = OC_6H_4Cl-*p*
23 R^3 = $OC_6H_4NO_2$-*p*

catalytic reactions were the α- and β-alcohols **18** and **26**.[12,13] The α-alcohol **18** was obtained by reduction of the corresponding ketone **15**. The β-ketone was prepared in two

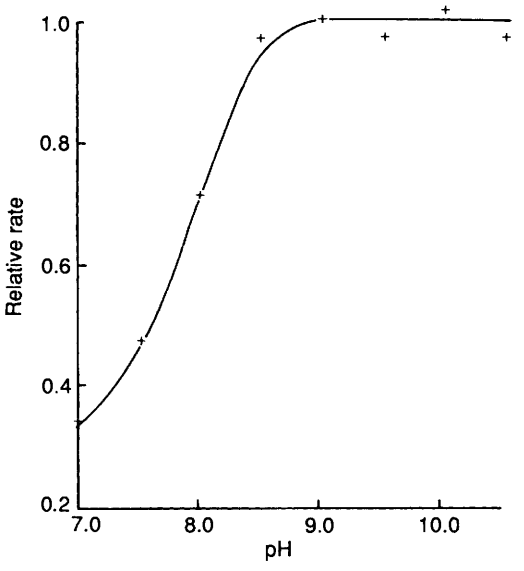
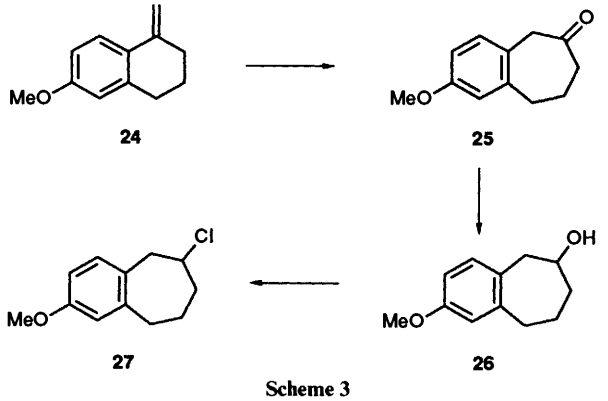


Fig. 2 Rate of hydrolysis of **23** by C3 expressed relative to the rate of hydrolysis at pH 8.5

steps from 5-methoxy-α-tetralone *via* the α-methylenetetralin **24** (Scheme 3). Treatment of the β-alcohol with thionyl chloride



Scheme 3

afforded the chloro derivative **27** but the α-alcohol underwent dehydration affording the alkene **19**.[12] A number of further compounds were synthesised as potential haptens for the production of antibodies and these led to further compounds that were used to probe the molecular recognition ability of the antibodies isolated. The relevant compounds **28–31** were prepared from known intermediates[7,8] using similar methods to those outlined above.

Preparation and Purification of Antibodies.—In order to raise antibodies and to assay their binding affinities, the hapten **6** was coupled *via* a butanoic acid linking arm to transferrin and bovine serum albumin. The antibodies were prepared by immunisation of NZB/Balb c F1 female mice and cloned using hybridoma technology as described elsewhere.[9] The antibodies were carefully purified and the purity checked by gel electrophoresis. Two clones of antibodies, C3 and C5, were found to bind strongly to the transferrin conjugate of the hapten. C3 was identified to belong to the IgG3 subtype and had a K_D 2.35 ± 0.11 × 10^{-8} mol dm^{-3} and C5 of the IgG2b subtype with a K_D of 1.89 ± 0.48 × 10^{-8} mol dm^{-3}.

Catalysis by C3.—Antibodies C3 and C5 were investigated for their ability to catalyse cyclisation reactions leading to benzocycloheptenones. Incubations were carried out typically with 0.05–0.25 mmol dm^{-3} substrate and 4.2 μmol dm^{-3} antibody at pH 8.5. Not surprisingly, the acids **11** and **12** were

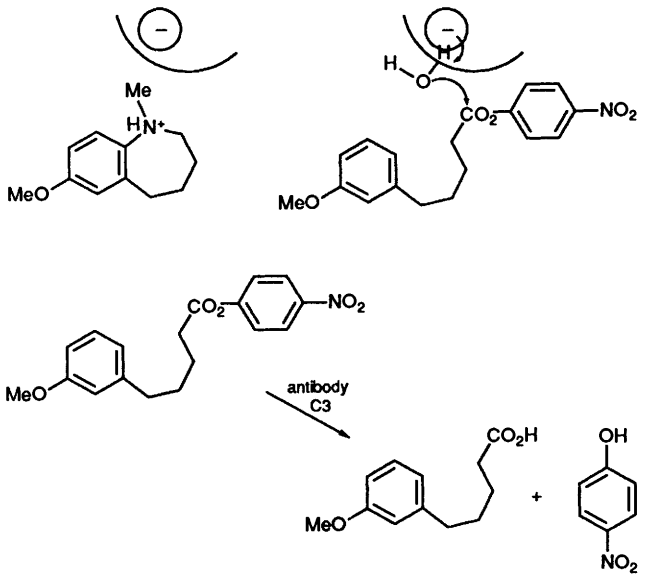


Fig. 3 Relationship of hapten structure and binding to the observed hydrolysis reaction catalysed by H11

unchanged. We therefore investigated a series of esters of increasing reactivity **20**–**23**. With antibody C5, no reactions of any kind were detected by HPLC. C3 also failed to catalyse the cyclisation reaction and also had no effect on the ethyl, ethylthio, or 4-chlorophenyl esters. However, when the 4-nitrophenyl ester **23** was introduced as substrate, the reaction mixture became intensely yellow indicating that the ester was undergoing hydrolysis. The products of the reaction were identifed as 4-nitrophenol and the acid **11** by UV spectroscopy and HPLC. A control experiment in which C3 was omitted developed a yellow colour only on incubation overnight. It therefore seemed that C3 was a catalyst for hydrolysis of the 4-nitrophenyl ester **23** and the mechanism of catalysis was investigated.

Firstly, it was shown that C3 catalyses hydrolysis according to Michaelis–Menten kinetics (Fig. 1). At pH 8.5, k_{cat} was determined to be 2.4 s^{-1} and K_m 0.4 mmol dm^{-3} giving a value of 6000 dm^3 mol^{-1} s^{-1} for k_{cat}/K_m indicating that C3 is operating as a respectably effective catalyst for this (trivial) reaction. Further, the rate constant of the uncatalysed solvolysis reaction under the same conditions was determined to be 6.9×10^{-7} s^{-1} which implies a rate enhancement of 3.5×10^6. The reaction was shown to be pH dependent (Fig. 2) with a plateau of maximum rate above pH 8. This result implies that an ionisable group of pK *ca.* 7 could be involved in the reaction, and chemical modification experiments were carried out in an attempt to identify the significant functional group.

To investigate the involvement of cysteine residues, antibody C3 was incubated with 4-hydroxymercuribenzoate; although there was an increase in absorbance at 252 nm, indicating the formation of an S–Hg bond, the catalytic activity of C3 was not disturbed. The significance of tyrosine residues was examined using tetranitromethane; in this case, no reaction between C3 and tetranitromethane was observed as indicated by the absence of absorbance at 428 nm. Iodoacetamide was used to probe the role of histidine residues; again, no loss of activity for hydrolysis was observed after modification. The only chemical modification that caused catalytic activity to be lost was incubation with glycine ethyl ester in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; in this case, the hydrolysis activity was completely inhibited. This result does not implicate a single residue in catalysis but it does suggest that a carboxylic acid has an important role. In view of the pH dependence of the reaction, it is reasonable to suggest that the carboxylate anion is functioning as a general base.

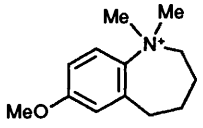
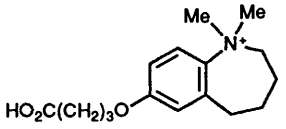
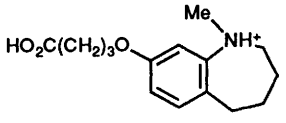
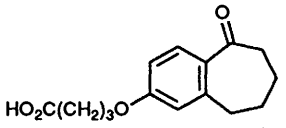
The significance of general base catalysis was further investigated by measuring the rate of reaction catalysed by C3 in deuterium oxide solution (Fig. 1). At pD 8.5, both K_m and v_{max} were found to be reduced leading to isotope effects $K^H_m/K^D_m = 1.6$ and $v^H_{max}/v^D_{max} = 2.5$. Both isotope effects were determined in a pH range in which the rate of reaction is not dependent upon pH. The small isotope effect on K_m has little diagnostic significance and may simply reflect a summation of the differences in hydrogen bonding between water and deuterium oxide. The larger isotope effect on v_{max} is consistent with general base catalysis and is comparable with isotope effects found in the hydrolysis of 4-nitrophenyl carbonate.[14]

Bearing in mind that an antibody raised to an ammonium hapten catalysed an elimination,[5] the ability of C3 and C5 to catalyse other reactions in which general base catalysis might play a role was investigated. Following Shokat[5] *et al.*, the ability of the antibodies to catalyse elimination of chloride from the 6-chlorobenzocycloheptene **27** was investigated but no conversion into the conjugated alkene **19** was observed. Neither antibody was able to catalyse the exchange of protium for deuterium in the ketones **15** and **25** and the catalysis of hydrolysis by C3 was not inhibited by incubation with the 5,6-epoxybenzocycloheptene. The catalytic activity of C3 therefore seems limited to the hydrolysis of 4-nitrophenyl ester **23**.

Molecular Recognition by C3 and C5.—Antibodies C3 and C5 bind the transferrin conjugates of the hapten **6** with virtually identical affinity but have very different chemical properties. Antibody C3 accidentally turned out to be an effective catalyst for one specific type of ester. It is easy to see how the hapten structure might lead to an antibody with the ability to hydrolyse **23** if the substrate adopts a closed conformation (Fig. 3); the general base region induced by the positive charge of the hapten would thus be placed near to the carbonyl group of the ester. It would have been expected, however, that such an arrangement would lead to hydrolysis of other esters, particularly the *p*-chlorophenyl ester **22**. The selectivity of C3 for the *p*-nitrophenyl group may therefore indicate that an adventitious binding site that favours *p*-nitrophenyl groups exists near to the general base site. We have argued elsewhere that hydrolysis by antibodies is to be expected in general;[4] other antibodies may therefore hydrolyse **23**. It is also possible to argue that other binding modes contribute to hydrolytic activity. Further studies will be required to investigate these questions.

The difference in properties of the two antibodies C3 and C5, however, must be related to their detailed molecular structures at the general base site and the parallel region in C5. With a series of molecules related to the hapten available from the syntheses of the haptens, it was possible to investigate the differences between C3 and C5 by competitive ELISA measurements. The experimental methods for this work have been published elsewhere[9] and a summary of the results is shown in Table 1. The data quoted are the percentage inhibition of binding of hapten–transferrin conjugate by the compounds listed. Some similarities and many clear differences betwen the two antibodies can be discerned. Both antibodies bind the hapten analogues bearing the short linking arms **6**, **29** and **30** as would be expected from the origin of the antibodies. On the other hand, C3 shows its greatest affinity for molecules bearing a positive charge (**4**·HCl, **6**, **28**–**30**). Further, C5 shows contrasting and virtually complementary behaviour. It has significant affinity for neutral molecules such as the alkene **19** and the ketones **15** and **16** in addition to accepting the positively charged molecules related to the hapten. The pK_a of the amino group of the methoxy analogue **3** hapten **6** was determined to be 5.43 ± 0.07 and, therefore, only a small proportion would be protonated at physiological pH. It may be that C3 arose from

Table 1 Affinities of analogues of hapten to C3 and C5 measured by competitive ELISA

	Compd. no.	% Binding to hydrolytic antibody (C3)	% Binding to non-hydrolytic antibody (C5)
	6	100	100
	2·HCl	122	88
	4	2	76
	4·HCl	80	82
	15	12	122
	16	14	120
	21	28	50
	18	4	80
	26	6	82
	19	18	116
	**28**	104	88
	**29**	107	142
 Cl⁻	**30**·HCl	124	104
	**31**	10	60

recognition of a protonated molecule and C5 from a molecule of free base. This would imply that C3 interacts with a positively charged hapten through an ionic group, probably a carboxylate. In contrast, C5 may respond through a non-ionic hydrogen bonding residue which permits the access of non-polar molecules such as the alkene **19** and polar molecules such as the amines, alcohols and ketones. The ability of the biological antibody producing system to provide variety and hence chemical selectivity is elegantly illustrated by these contrasts. Further work will be required to identify the residues involved and to place the affinities of the analogues tested on a firm thermodynamic basis.

Experimental

^{1}H NMR spectra were recorded on a Perkin-Elmer R32 instrument operating at 90 MHz. Except where otherwise stated, the solvent was $CDCl_3$ and tetramethylsilane (TMS) was used as internal standard.

2,3,4,5-Tetrahydro-7-hydroxy-1-methyl-1-benzazepine **4**.—2,3,4,5-Tetrahydro-7-methoxy-1-methyl-1-benzazepine[8] **3** (6.80 g, 35.60 mmol)[9] in dry dichloromethane (80 cm^3) was added dropwise to boron tribromide (50 cm^3) in dry dichloromethane (80 cm^3) under nitrogen, at −80 °C. After the addition the stirred mixture was left to rise to room temperature overnight. Water (100 cm^3) was added dropwise and then the mixture was washed with dichloromethane. The water layer was neutralised with sodium hydrogen carbonate, then a little extra $NaHCO_3$ was added when the effervescence died down. The pale yellow solid material was filtered off, washed with water and dried to give the *title compound* **4** (5.50 g, 87%), m.p. 146–149 °C (Found: C, 74.7; H, 8.4; N, 7.4%; M$^+$, 177.1140. $C_{11}H_{15}NO$ requires C, 74.6; H, 8.5; N, 7.9%; *M*, 177.1154); ν_{max}/cm^{-1} 600 (aromatic, C=C), 820 and 850 (aromatic); δ_H[$(CD_3)_2SO$] 8.8 (1 H, s, OH, exch.), 6.9–6.5 (3 H, m, ArH), 2.9–2.6 (7 H, m, 2 × CH_2 and NCH_3) and 1.9–1.4 (4 H, m, 2 × CH_2).

Ethyl 4-(2,3,4,5-Tetrahydro-1-methyl-1-benzazepin-7-yloxy)-butanoate **5**.—2,3,4,5-Tetrahydro-7-hydroxy-1-methyl-1-benzazepine (1.038 g, 5.864 mmol) was added portionwise to a stirred suspension of potassium carbonate (5.011 g) in dry acetone (200 cm^3). Ethyl 4-iodobutyrate (4.089 g, 16.90 mmol) in dry acetone (50 cm^3) was added dropwise. The reaction mixture was refluxed for 48 h. The cooled reaction mixture was filtered, the solvent removed under reduced pressure and the residual brown oil chromatographed on neutral alumina using ethyl acetate–light petroleum (b.p. 60–80 °C) (1:3). The product was obtained as pale yellow oil, which was distilled (Kugelrohr) to give the *title compound* **5** as a colourless oil (1.443 g, 85%) (Found: C, 69.9; H, 5.7; N, 4.7. $C_{17}H_{25}NO_3$ requires C, 70.1; H, 8.6; N, 4.8%); ν_{max}/cm^{-1} (C=O), 1050 (C–OMe) and 810 (aromatic); δ_H 6.90–6.65 (3 H, m, ArH), 4.2–4.1 (2 H, q, CH_3CH_2O), 4.0–3.9 (2 H, t, CH_2OAr), 2.90–2.75 [7 H, m and s, $(CH_2)_2$ and OCH_3] 2.55–2.50 (2 H, t, CH_2), 2.15–2.00 (2 H, q, CH_2), 1.80–1.70 (2 H, m, CH_2), 1.65–1.50 (2 H, m, CH_2) and 1.30–1.20 (3 H, t, CH_3CH_2O).

4-(2,3,4,5-Tetrahydro-1-methyl-1-benzazepin-7-yloxy)butanoic Acid Hydrochloride **6**.—Ethyl 4-(2,3,4,5-tetrahydro-1-methyl-1-benzazepin-7-yloxy)butanoate (1.3 g, 4.467 mmol) was dissolved in ethanol (15 cm^3). Sodium hydroxide (1.00 g) in

water (35 cm^3) was added and the mixture then refluxed for 4 h. The cooled solution was extracted with diethyl ether, then the water layer acidified with conc. HCl. Water was removed under reduced pressure to yield a light brown thick oil in quantitative yield. Dry acetone was added and gave the *title compound* **6** as white crystals. After two recrystallisations from acetone, the yield was 1.020 g (76%), m.p. 173–175 °C (Found: C, 60.5; H, 7.8; Cl, 12.0; N, 4.7. $C_{15}H_{22}ClNO_3$ requires C, 60.1; H, 7.4; Cl, 11.8; N, 4.68%) (Found: M^+ – HCl, 263.1540. $C_{15}H_{21}ClNO_3$ requires *M*, 263.1521); ν_{max}/cm^{-1} 2600–2700br (H–N–Me), 1720 (CO), 1030 (C–OMe) and 760 (aromatic); $\delta_H(CD_3OD)$ 8.05–8.00 (1 H, d, ArH), 6.8–6.7 (2 H, m, ArH), 4.1–4.0 (2 H, t, CH_2), 3.7–3.4 (2 H, m, CH_2), 3.2–2.9 (5 H, m, CH_2 and NCH_3), 2.6–2.5 (2 H, t, CH_2) and 2.3–2.0 (6 H, m, 3 × CH_2).

Ethyl 5-(3-*Methoxyphenyl*)*pentanoate* **20**.[10]—5-(3-Methoxyphenyl)pentanoic acid (1.220 g, 6.280 mmol), potassium carbonate (3.550 g) and iodoethane (4.010 g, 25.647 mmol) were refluxed in dry acetone (69 cm^3) for 48 h. The reaction mixture was cooled to room temperature, filtered and solvent removed under reduced pressure. The product was chromatographed over neutral alumina, using ethyl acetate–light petroleum 1:3 to afford the title compound **20** as a colourless oil (1.293 g, 93%); ν_{max}/cm^{-1} 1730 (C=O), 1600, 1580 (C=C), 1040 (OMe), 700 and 780 (aromatic); δ_H 7.1–7.3 (1 H, m, ArH), 6.65–6.85 (3 H, m, ArH), 4.0–4.3 (2 H, q, CH_2), 3.8 (3 H, s, OMe), 2.5–2.8 (2 H, t, CH_2), 2.2–2.4 (2 H, t, CH_2), 2.5–2.8 (4 H, p, 2 × CH_2) and 1.2–1.4 (3 H, t, CH_3).

S-*Ethyl* 5-(3-*Methoxyphenyl*)*pentanethioate* **21**.—5-(3-Methoxyphenyl)pentanoic acid **11** (0.630 g, 3.025 mmol) was dissolved in dry dichloromethane (10 cm^3), followed by the addition of 1,1′-carbonyldiimidazole (0.423 g, 2.609 mmol) at room temperature with stirring. After the effervescence stopped, (*ca.* 10 min) ethanethiol (2 cm^3) was added and the reaction mixture was left at room temperature overnight. Solvent was removed under reduced pressure at room temperature and the crude product was chromatographed using neutral alumina and ethyl acetate–light petroleum (1:2). The *product* **21** was obtained as a pale yellow liquid, b.p. 170 °C (0.3 Torr) (0.724 g, 95%) (Found: C, 66.9; H, 7.9; S, 12.9%; M^+, 252.1178. $C_{14}H_{20}O_2S$ requires C, 66.6; H, 8.0; S, 12.7%; *M*, 252.1184); ν_{max}/cm^{-1} 1680 (C=O), 1600 (C=C), 1040 (OMe), 700 and 770 (aromatic); δ_H 7.1–7.3 (1 H, m, ArH), 6.6–6.85 (3 H, m, ArH), 3.9 (3 H, s, OMe), 2.75–3.0 (2 H, q, CH_2), 2.45–2.7 (4 H, m, 2 × CH_2), 1.5–1.8 (4 H, m, 2 × CH_2) and 1.15–1.35 (3 H, t, CH_3).

4-*Chlorophenyl* 5-(3-*Methoxyphenyl*)*pentanoate* **22**.—5-(3-Methoxyphenyl)pentanoic acid (0.323 g, 1.551 mmol) and thionyl chloride (91 cm^3) were refluxed for 3 h. Thionyl chloride was removed under reduced pressure to give a pale yellow oil (0.352 g, 100%). *p*-Chlorophenol (0.209 g, 1.626 mmol) was added to dry pyridine (0.260 g, 3.287 mmol). The acid chloride in dry dichloromethane (10 cm^3) was added dropwise at room temperature to the phenol–pyridine mixture with stirring which was continued overnight at room temperature. The solvent was removed under reduced pressure at room temperature and the residue was chromatographed over neutral alumina, using ethyl acetate–light petroleum (1:5). The *product* **22** (0.349 g, 71%) was obtained as a colourless oil, b.p. 220 °C (0.3 Torr) which solidified on storage at 4 °C (Found: C, 67.3; H, 5.75%; M^+, 318.1023. $C_{18}H_{19}ClO_3$ requires C, 67.8; H, 6.0%; *M*, 318.1023); ν_{max}/cm^{-1} 1760 (C=O), 1600 (C=C), 1020, 1050 (OMe), 700, 780 and 850 (aromatic); δ_H 6.7–7.4 (8 H, m, ArH), 3.8 (3 H, s, OMe), 2.5–2.8 (4 H, m) and 1.7–2.0 (4 H, m).

4-*Nitrophenyl* 5-(3-*Methoxyphenyl*)*pentanoate* **23**.—To 5-(3-methoxyphenyl)pentanoic acid (0.460 g, 2.209 mmol) in dry dichloromethane (15 cm^3), thionyl chloride (3 cm^3) was added and the mixture refluxed for 3 h. The solvent was removed under reduced pressure to give a pale yellow oil (quantitative yield) which was dissolved in dry dichloromethane (10 cm^3) and added to a mixture of *p*-nitrophenol (0.342 g, 2.458 mmol) and dry pyridine (0.21 g, 2.668 mmol) in dry dichloromethane (10 cm^3) at room temperature with stirring. The reaction mixture was stirred at room temperature overnight after which the solvent was removed under reduced pressure. The reaction mixture was then chromatographed using neutral alumina and ethyl acetate–light petroleum (1:3 v/v) to give the *product* **23** as a pale yellow oil, which crystallised on storage (0.565 g, 80%), m.p. 50–52 °C. It was recrystallised from light petroleum m.p. 54–56 °C (Found: C, 65.7; H, 5.9; N, 4.2. $C_{18}H_{19}NO_5$ requires C, 65.65; H, 5.8; N, 4.25%); ν_{max}/cm^{-1} 1750 (C=O), 1600 (C=C), 1040 (OMe), 700, 750 and 800 (aromatic); δ_H 8.15–8.35 (2 H, d, ArH), 7.1–7.4 (3 H, m, ArH), 6.7–6.9 (3 H, m, ArH), 3.8 (3 H, s, OMe), 2.5–2.8 (4 H, 2 × CH_2) and 1.5–1.9 (4 H, m, 2 × CH_2).

Conjugation of 4-(2,3,4,5-*Tetrahydro*-1-*methyl*-1-*benzazepin*-7-*yloxy*)*butanoic Acid Hydrochloride to BAS and Tf*: **I** *and* **II**.—The carboxylic acid hydrochloride **6** (20 mg) was dissolved in DMF (200 mm^3). *N*-Hydroxysuccinimide (10 mg) was dissolved in DMF (200 mm^3). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (10 mg) was dissolved in DMF (200 mm^3). The above solutions were mixed and left at room temperature (protected from light) for 18 h. The resulting mixture was then split into two 300 mm^3 aliquots (A and B).

The following solutions were prepared: (C) human transferrin (10 mg) in 2 cm^3 0.9% NaCl (pH 6.5); (D) BSA (8 mg) in 2 cm^3, 0.9% NaCl (pH 6.5). To solution (A) was added (C) with stirring. To solution (B) was added (D) with stirring. Both A + C and B + D were stirred at 4 °C for 4 h. They were then applied separately to a PD10 Sephadex column and eluted with phosphate buffered saline (PBS) (pH 7.5), 3 cm^3 fractions were collected, the A_{280} measured, and fractions with high optical density were collected.

8,9-*Dihydro*-2-*methoxybenzocyclohepten*-6-*one* **25**.[12]—Thallium(III) nitrate·$3H_2O$ (4.50 g, 10 mmol) and methanol (40 cm^3) were cooled to 0 °C. 1,2,3,4-Tetrahydro-6-methoxy-1-methylenenaphthalene **24** (1.742 g, 10 mmol) was added in small portions with stirring. After 1 min the reaction mixture was diluted with chloroform (30 cm^3), filtered and the filtrate neutralised with aqueous sodium hydrogen carbonate. The organic layer was washed with water, dried (Na_2SO_4) and concentrated under reduced pressure to give the ketone **25** (1.95 g, 100%); δ_H 7.0–7.1 (1 H, d, ArH), 6.6–6.8 (2 H, m, ArH), 3.8 (3 H, s, OMe), 3.65 (2 H, s, CH_2), 2.8–3.0 (2 H, t, CH_2), 2.45–2.75 (2 H, t, CH_2) and 1.85–2.15 (2 H, q, CH_2).

6-*Chloro*-6,7,8,9-*tetrahydro*-2-*methoxybenzocycloheptene* **27**.[12]—6,7,8,9-Tetrahydro-6-hydroxy-2-methoxybenzocycloheptene **26**[13] (0.423 g, 2.200 mmol), dry dichloromethane (10 cm^3) and thionyl chloride (3 cm^3) were refluxed for 3 h. Solvent was removed under reduced pressure to leave a light brown oil. This was distilled (Kugelrohr) 150 °C (0.1 mmHg) to give the title compound **27** as a pale yellow oil (0.357 g, 77%) (Found: Cl 16.9. $C_{12}H_{15}ClO$ requires Cl, 16.8%); ν_{max}/cm^{-1} 1580, 1610 (C=C), 1040 (OMe), 700 and 820 (aromatic); δ_H 6.6–7.2 (3 H, m, ArH), 3.8 (3 H, s, OMe), 3.2 (1 H, d), 2.7–2.8 (2 H, m) and 1.7–2.2 (3 H, m).

2,3,4,5-*Tetrahydro*-7-*methoxy*-1-*methyl*-1-*benzazepine Methiodide* **28**.—2,3,4,5-Tetrahydro-7-methoxy-1-methyl-1-benzazepine **3**[3,15] (0.786 g, 4.434 mmol), sodium hydroxide (5%; 20 cm^3) and iodomethane (11.12 g, 78.343 mmol) were refluxed for 18 h. The reaction mixture was cooled to room temperature and then extracted with chloroform. The organic solvent was

removed under reduced pressure and the product recrystallised from chloroform–diethyl ether to give the *title compound* **28** as a white crystalline solid (1.105 g, 78%), m.p. 179–181 °C (Found: C, 46.5; H, 5.5; I, 37.9; N, 4.0. $C_{13}H_{20}ION$ requires C, 46.85; H, 6.05; I, 38.1; N, 4.2%); ν_{max}/cm^{-1} 1600, 1500 (C=C), 1030 (OMe), 810 and 900 (aromatic); δ_H 7.95 (1 H, d, aromatic), 6.9–7.0 (1 H, dd, aromatic), 6.85 (1 H, d, aromatic), 4.25 (2 H, t), 4.0 (6 H, s, 2 × Me), 3.85 (3 H, s, OMe), 3.2 (2 H, t, CH_2), 2.4 (2 H, p, CH_2) and 1.9–2.0 (2 H, p, CH_2).

Ethyl 4-(2,3,4,5-*Tetrahydro*-1-*methyl*-1-*benzazepin*-7-*yloxy*)-*butanoate Methiodide*.—Ethyl 4-(2,3,4,5-tetrahydro-1-methyl-1-benzazepin-7-yloxy)butanoate **5** (0.093 g, 0.319 mmol) was dissolved in an excess of methyl iodide. The solution was protected from the light and left at room temperature for 6 days. The solvent was removed under reduced pressure to give a thick oil. Crystallisation from acetone gave the *title compound* as a pale yellow solid, m.p. 117–119 °C (0.132 g, 96%) (Found: C, 49.6; H, 6.5; I, 29.7; N, 3.2. $C_{18}H_{28}INO_3$ requires C, 49.9; H, 6.5; I, 29.3; N, 3.25%); ν_{max}/cm^{-1} 1710 (CO_2Et), 1690 and 820 (aromatic); δ_H 7.9–8.0 (1 H, d, ArH), 6.9 (1 H, dd, ArH), 6.8 (1 H, d, ArH), 4.0–4.3 (4 H, t and q, 2 × CH_2), 3.95 (6 H, s, OMe), 3.0–3.2 (2 H, t, CH_2), 2.3–2.6 (4 H, dt, 2 × CH_2), 1.8–2.2 (4 H, m, 2 × CH_2), 1.5–1.7 (2 H, t, CH_2) and 1.2–1.4 (3 H, t, CH_3).

4-(2,3,4,5-*Tetrahydro*-1-*methyl*-1-*benzazepin*-7-*yloxy*)*butanoic Acid Methiodide* **29**.—Ethyl 4-(7-*N*-methyl-2,3,4,5-tetrahydro-1-benzazepinyloxy)butanoate methiodide (0.17 g, 0.247 mmol) was dissolved in ethanol (10 cm^3). Sodium hydroxide (0.50 mmol) was added and the mixture refluxed for 1 h. The cooled solution was neutralised with dil. HCl. The solvent was evaporated under reduced pressure. The solid material was dissolved in dry acetone, filtered, then solvent was removed to give a thick yellow oil (90 mg, 90%). Recrystallisation from dry acetone gave the *title compound* **29** as off-white crystals, m.p. 148–150 °C (Found: C, 47.6; H, 5.8; I, 31.1; N, 3.6. $C_{16}H_{24}INO_3$ requires C, 47.4; H, 6.0; I, 31.3; N, 3.5%); ν_{max}/cm^{-1} 1720 (CO_2H) 720, 770, 790 and 810 (aromatic); $\delta_H(CD_3OD)$ 7.8 (1 H, d, ArH), 7.0 (2 H, m, ArH), 4.1 (2 H, t, CH_2), 3.9 (2 H, t, CH_2), 3.6 (6 H, s, 2 × Me), 3.2 (2 H, t, CH_2), 2.5 (2 H, t, CH_2), 2.3 (2 H, p, CH_2), 2.1 (2 H, p, CH_2) and 1.8–1.9 (2 H, p, CH_2).

2,3,4,5-*Tetrahydro*-1-*methyl*-1-*benzazepin*-7-*ol* **4**.—2,3,4,5-Tetrahydro-8-methoxy-1-methyl-1-benzazepine[8] (3.50 g, 18.30 mmol) in dry dichloromethane (40 cm^3), was added dropwise to boron tribromide (25 cm^3) in dry dichloromethane (45 cm^3), under N_2, while the temperature was maintained at −80 °C. After the addition was complete, the stirred reaction mixture was allowed to reach room temperature, overnight. Water (50 cm^3) was added slowly and then the reaction mixture was extracted with water (550 cm^3). The water layer was separated and sodium hydrogen carbonate added to it until the effervescence stopped. The solid pale yellow *product* **4** was filtered off, washed with water and dried (2.70 g, 83%). It was chromatographed on neutral alumina, eluting with diethyl ether–light petroleum (1:1), v/v, and then crystallised from diethyl ether, m.p. 113–115 °C (Found: C, 74.1; H, 8.4; N, 7.5%; M^+, 177.1158. $C_{11}H_{15}NO$ requires C, 74.6; H, 8.5; N, 7.5%; *M*, 177.1154%); ν_{max}/cm^{-1} 3000–3200br (OH), 1590, 1610 (aromatic), 980 (O–OCH_3), 810 and 860 (aromatic); δ_H 7.8 (1 H, s, OH exch.), 6.9–6.8 (1 H, d, ArH), 6.5–6.2 (2 H, m, ArH), 2.95–2.75 (5 H, t and s, 2 × CH_2 and NMe), 2.70–2.55 (2 H, t, CH_2) and 1.9–1.4 (4 H, m, 2 × CH_2).

Ethyl 4-(2,3,4,5-*Tetrahydro*-1-*methyl*-1-*benzazepin*-1-*yloxy*)-*butanoate Methiodide* (*Ethyl Ester of* **30**).—The foregoing compound **4** (1.02 g, 5.650 mmol), potassium carbonate (5.10 g), sodium iodide (4.01 g), and ethyl 4-bromobutanoate (5.03 g) in dry acetone (300 cm^3) were refluxed for 48 h. The cooled solution was filtered and the solvent removed under reduced pressure (using Kugelrohr apparatus). It was chromatographed on neutral alumina and eluted with ethyl acetate–light petroleum (1:4, v/v), to give a pale yellow oil (1.60 g, 97%) (Found: C, 70.2; H, 8.4; N, 4.8. $C_{17}H_{25}NO_3$ requires C, 70.05; H, 8.65; N, 4.8%); ν_{max}/cm^{-1} 1730 (C=O), 1610, 1580 (aromatic), 1060 (C–O–C), 800 and 850 (aromatic); δ_H 6.98–6.95 (1 H, d, ArH), 6.48–6.47 (1 H, d, ArH), 6.38–6.34 (1 H, dd, ArH), 4.18–4.10 (2 H, q, CH_3CH_2O), 2.92–2.89 (2 H, t, CH_2), 4.00–3.95 (2 H, t, CH_2O), 2.84 (3 H, s, NMe), 2.73–2.68 (2 H, t, CH_2), 2.54–2.48 (2 H, t, CH_2), 2.12–2.04 (2 H, q, CH_2), 1.8–1.7 (2 H, m, CH_2), 1.6–1.5 (2 H, m, CH_2) and 1.3–1.2 (3 H, t, CH_3CH_2).

4-(2,3,4,5-*Tetrahydro*-1-*methyl*-1-*benzazepin*-7-*yloxy*)*butanoic Acid Hydrochloride* **30**.—The above ester (1.60 g, 5.498 mmol) ethanol (15 cm^3), sodium hydroxide (1.0 g) and water (35 cm^3) were refluxed for 4 h. The cooled solution was extracted with diethyl ether, then the water layer acidified with conc. HCl. The solvent was removed under reduced pressure to yield a light brown oil and solid (NaCl) which was filtered off. The product was washed with dry acetone and the latter removed under reduced pressure to give a pale yellow oil which crystallised readily. The product was washed with dry acetone and filtered. Recrystallisation from acetone afforded the desired *compound* **30** as a white crystalline material (1.10 g, 73%), m.p. 167–169 °C (Found: C, 60.2; H, 7.1; Cl, 11.9; N, 4.6. $C_{15}H_{22}ClNO_3$ requires C, 60.1; H, 7.4; Cl, 11.82; N, 4.7%); ν_{max}/cm^{-1} 1740 (C=O), 1580, 1610 (aromatic C=C), 1055 (C–OMe), 790 and 850 (aromatic); δ_H 14.0–13.5 (1 H, br, OH exch.), 7.7–7.6 (1 H, br, ArH), 7.2–7.2 (1 H, d, ArH), 6.8 (1 H, d, ArH), 4.10–4.05 (2 H, t, CH_2), 3.7–3.3 (4 H, m, 2 × CH_2), 3.2 (3 H, s, NMe), 3.1–2.9 (2 H, m, CH_2), 2.6–2.5 (2 H, t, CH_2) and 2.3–2.1 (4 H, m, 2 × CH_2).

Ethyl 4-(6,7,8,9-*Tetrahydro*-5-*oxobenzocyclohepten*-2-*yloxy*)-*butanoate* (*Ethyl Ester of* **31**).—6,7,8,9-Tetrahydro-2-hydroxybenzocyclohepten-5-one (1.00 g, 5.682 mmol) was added portionwise to a stirred suspension of potassium carbonate (5.10 g) in dry acetone (100 cm^3). Ethyl 4-bromobutanoate (4.22 g, 21.634 mmol) in dry acetone (50 cm^3) was added dropwise followed by sodium iodide (3.45 g, 23.015 mmol). The reaction mixture was refluxed for 24 h then cooled and the solvent removed under reduced pressure. The product was purified using a neutral alumina column, then a Kugelrohr apparatus was used to remove excess ethyl 4-iodobutyrate. The *product* was obtained as a colourless oil (1.36 g, 83%) (Found: C, 69.7; H, 6.85. $C_{17}H_{22}O_4$ requires C, 70.3; H, 7.65%); δ_H 7.7–7.8 (1 H, d, ArH), 6.6–6.9 (2 H, m, ArH), 4.0–4.4 (4 H, q and t), 2.4–3.0 (6 H, m), 2.0–2.3 (2 H, q), 1.7–2.0 (4 H, m) and 1.2–1.4 (3 H, t, Me).

4-(6,7,8,9-*Tetrahydro*-5-*oxobenzocyclohepten*-2-*yloxy*)*butanoic Acid* **31**.—Ethyl 4-(6,7,8,9-tetrahydro-5-oxobenzocyclohepten-2-yloxy)butanoate (0.01 g) was dissolved in ethanol (3 cm^3) and sodium hydroxide (7 cm^3, 7.5 mmol) was added to the stirred solution at room temperature. The mixture was heated on a steam bath for 5 min after which dilute HCl was added until the solution became acidic. The organic solvent was removed under reduced pressure and the precipitated *carboxylic acid* **31** was filtered off and washed with water to give white crystalline material (0.070 g, 77%), m.p. 82–84 °C (Found: C, 69.3; H, 7.0. $C_{15}H_{18}O_4$ requires C, 68.7; H, 6.9%); ν_{max}/cm^{-1} 1730 (C=O ester), 1630 (C=O), 1600 (C=C), 730, 760, 800 and 830 (aromatic); $\delta_H(CDCl_3)$ 7.8 (1 H, d, ArH), 6.8 (1 H, dd, ArH), 6.7 (1 H, d, ArH), 4.1 (2 H, t, CH_2), 2.9 (2 H, t, CH_2), 2.75 (2 H, t, CH_2), 2.6 (2 H, t, CH_2), 2.2 (2 H, p, CH_2) and 1.8–2.0 (4 H, m).

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