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Twisted α-Keto Amides as Transition-State Analogues for Acyl-Transfer Reactions: Synthesis of the Immunoconjugates[1]

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Abstract—Two new haptens were employed as antigens to elicit antibodies for acyl-transfer reactions. The haptens, both α-keto amides were designed to elicit antibodies which could twist potential substrates into a much more reactive conformation. The rationale for their design, synthesis, and immunization protocols will be discussed.

Introduction

Specific monoclonal antibodies, which belong to the immunoglobulin G subgroup (IgG), have been used to effectively catalyze various chemical reactions.[2–6] The first reports of antibody catalysis decribed the hydrolysis of aromatic esters and carbonates.[7–10] This was followed by communications, in which catalytic antibodies were shown to be specific for the hydrolysis of unactivated esters,[11] which included a meso diacetate,[12] as well as antibodies for the stereospecific hydrolysis of other alkyl esters.[13,14]

Mechanistic investigations of base-induced acyl-transfer reactions have revealed reaction pathway(s) that traverse through some form of tetrahedral transition-state. This occurring through the concerted attack of hydroxide ion on the electrophilic carbonyl carbon atom concomitant with the development of a negative charge on the carbonyl oxygen atom. Haptens, the essential element in obtaining catalytic antibodies have typically been designed to mimic this negatively charged, tetrahedral transition-state found within esterolytic reactions. The most successful haptens have utilized phosphonates for the catalytic antigenic determinant; its success has been attributed to its tetrahedral geometry, the phosphorus–oxygen bond length (typically 10–15 % longer than a carbon–oxygen bond) and the evenly distributed negative charge between the phosphorus–oxygen atoms.[15]

Phosphonates have been the most actively employed immunogens for obtaining antibody catalysts. Other types of transition-state analogues have also been used to elicit catalytic antibodies for hydrolytic reactions. In one example, Schultz *et al.* exploited a pepstatin[16] based approach in designing the hapten for the hydrolysis of *para*-nitrophenylacetate and *para*-nitrophenylcarbonate.[17] In a different methodology that we pioneered and termed "bait and switch", amino acid(s) are elicited within the antibody binding pocket to assist in the hydrolytic acyl-transfer reaction.[18,19] Overall these and other approaches have been highly successful. However, to overcome even more difficult acyl-transfer reactions (i.e. amide hydrolysis) either a larger array of antibodies must be sampled[20] and/or new hapten designs must be explored. It is this latter methodology which we have examined in this report.

X-ray crystal studies of both FK 506 and rapamycin[21] have shown that the keto and amide carbonyls are oriented orthogonally to one another with a dihedral angle of 95 degrees.[22] In addition, a recent study showed that α-keto amide of FK 506 functions as a twisted amide surrogate of a peptide substrate of FKBP rotamase.[23] In a separate study of the human α-thrombin inhibitor cyclotheonamide B, it was proposed that the α-keto group of cyclotheonamides might function as an electrophilic mimic of the ArgX scissile amide bond of thrombin substrates (see Figure 1).[24] Furthermore, model studies have delineated the susceptibility of highly strained amides to undergo facile hydrolysis.[25] Keeping these studies in mind, we have investigated the potential of α-keto amides as haptens in the procurement of monoclonal antibodies, which might catalyze acyl-transfer reactions. As a first step, we report the synthesis of two haptens (**1** and **2**) which contain this α-keto amide moiety subunit (see Figure 2). In addition, we detail the immunization process of these antigens and the obtainment of their perspective antibodies.

Results

The preparation of the hapten 1

5-[(4-Nitrophenyl)amino]-5-oxopentanoic acid[26] **3**, was refluxed in methanol using concentrated sulfuric acid as a catalyst to give methyl ester **4** (Scheme I). This ester was hydrogenated under 3 atm of hydrogen to afford methyl 5-

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Dedicated to Professor J. Bryan Jones on the occasion of his 60th birthday.

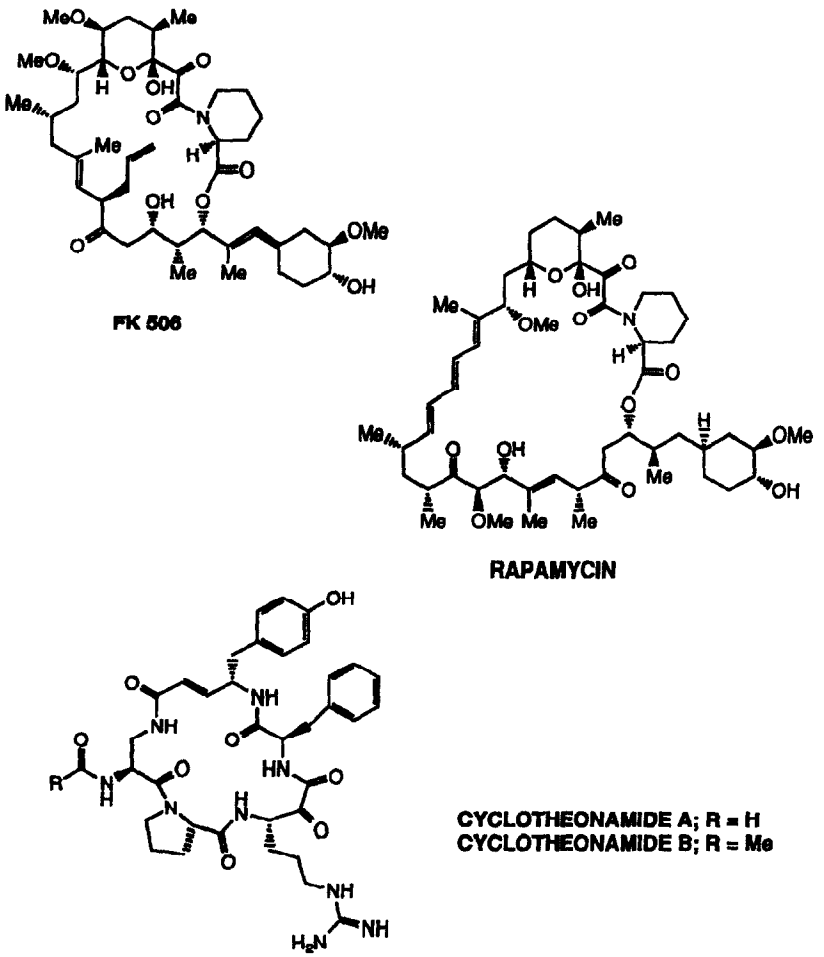


Figure 1. Structures of two immunophilin inhibitors: FK 506 and rapamycin and α-thrombin inhibitors: cyclotheonamide A and B.

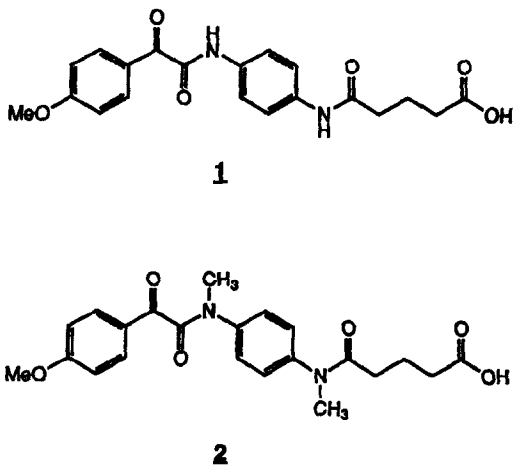


Figure 2. Structures of the haptens.

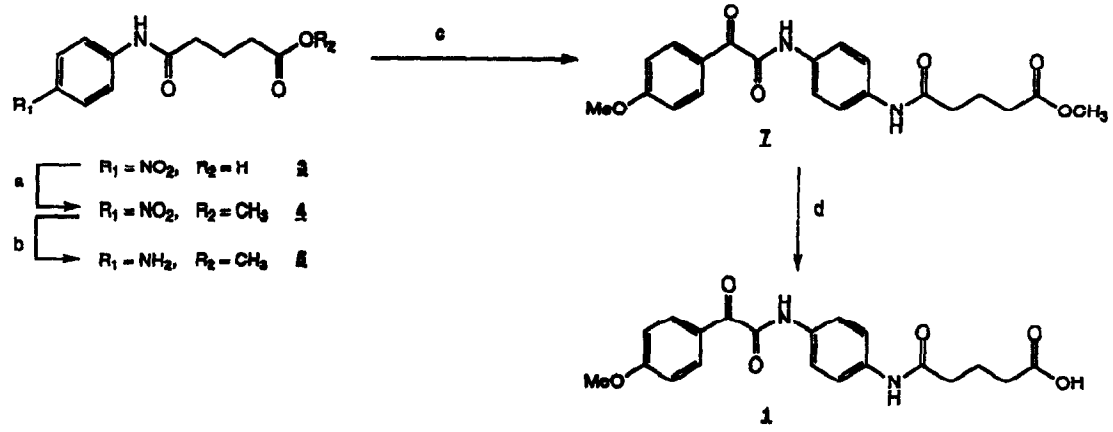
[(4-aminophenyl)amino]-5-oxo-pentanoate **5** in 86 % yield (three steps).

Several attempts to synthesize 4-methoxyphenylglyoxylic acid **6** were undertaken. Initially we investigated the benzylic oxidation employing either pyridinium dichromate[27] (PDC), pyridinium chlorochromate[28,29] (PCC) at room temperature or manganese (IV) dioxide[30] at 50 °C. However, both methods provided very poor yields of the 4-methoxyphenylglyoxylic acid (15, 14 and 26 %, respectively). The major product from these reactions was 4-methoxybenzaldehyde, its formation, we believe, occurs via an oxidative cleavage of the carbon–carbon bond of the cyclic chromium or manganese ester, respectively. In another attempt, the reaction of (±)-4-methoxymandelic acid with the catalytic oxidant tetra-*n*-propylammoniumperruthenate[31] (TPAP), using *N*-methylmorpholine *N*-oxide (NMO) as co-oxidant afforded 4-methoxybenzoic acid as a major product (92 %). The formation of this product can also be explained by the oxidative cleavage of the carbon–carbon bond of the five-member cyclic ruthenium ester. The successful synthesis of **6** was accomplished by the oxidation of (±)-4-methoxymandelic acid with potassium permanganate in $NaOH/H_2O$ at 0 °C, this then was found to proceed smoothly affording **6** in 83 % yield.[32–34]

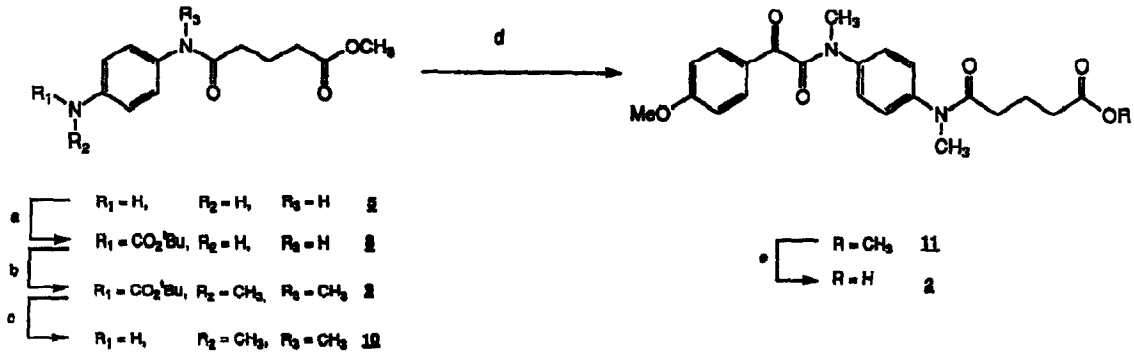
4-Methoxyphenylglyoxylic acid **6** was coupled to **5** using bis[2-oxo-3-oxazolidinyl]phosphinic chloride[35] (BOP-Cl) as an activating agent in triethylamine/CH_2Cl_2 to give 7 in good yield. The methyl ester was deprotected with $NaOH/H_2O$ affording hapten **1** in 78 % yield (last two steps).

The preparation of the hapten 2

Methyl 5-[(4-aminophenyl)amino]-5-oxopentanoate **5**, was *N*-protected as the *tert*-butyloxycarbonyl group using di-



Scheme I. (a) MeOH, cat. H_2SO_4, reflux, 2 h, 96 %; (b) 3 atm H_2, 10 % Pd/C catalyst, MeOH, rt, 1.5 h, 93 %; (c) 4-methoxyphenylglyoxylic acid **6**, BOP-Cl, TEA, CH_2Cl_2, 0 °C, 1 h, 85 %; (d) NaOH, H_2O, rt, 20 min, 92 %.



Scheme II. (a) (t-BuOCO)$_2$O, 1,4-dioxane, 85 °C, 12 h, 81 %; (b) 2.0 equiv. of MeI, KOBu-t, THF, rt, 45 min, 90 %; (c) trifluoroacetic acid, CH_2Cl_2, rt, 25 min; then TEA, CH_2Cl_2, 76 %; (d) 4-methoxyphenylglyoxylic acid **6**, BOP-Cl, TEA, CH_2Cl_2, rt, 36 h, 92 %; (e) NaOH, H_2O, 0 °C, 20 h, 76 %.

tert-butyldicarbonate[36,37] to give **8** in 81 % yield (Scheme II). Methylation of **8** using iodomethane as an alkylating agent and potassium *tert*-butoxide as a base proceeded in excellent yield to provide **9**. The *tert*-butyloxycarbonyl protecting group was removed by treatment of **9** with trifluoroacetic acid at room temperature to give compound **10** in a 55 % yield (3 steps). 4-Methoxyphenylglyoxylic acid **6** was coupled to **10** using BOP-Cl in TEA/CH_2Cl_2 to afford **11** in 92 % yield.[35] The hydrolysis of methyl ester **11** with NaOH/H_2O at 0 °C provided the hapten **2** in 76 % yield.

^{13}C NMR studies (DMF-d_7 or DMSO-d_6, 10 % D_2O, 1,4-dioxane as an internal standard) of the haptens **1** and **2** showed an absence of hydration within the α-keto appendage. Based on these findings it was anticipated that compounds **1** and **2** would exist in their keto forms during the immunization process rather than a *gem*-diol configuration.[38,39]

Activation, coupling, and the immunization

The α-keto amide haptens **1** and **2** were activated for coupling with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and *N*-hydroxysulfosuccinimide.[40–42] The activated haptens were coupled to keyhole limpet hemocyanin (KLH) and the immunoconjugates were used to immunize 129GIX$^+$ mice for the production of monoclonal antibodies.[43–45] Twenty-two monoclonal antibodies for the hapten **1** and 26 for the hapten **2** were shown by an enzyme-linked immunosorbent assay[46,47] (ELISA) to bind to the α-keto amide haptens of **1** and **2** conjugated to bovine serum albumin.

All 22 and 26 cell lines for the haptens **1** and **2**, respectively, were cloned and injected separately into mice for production of ascites fluid. Monoclonal antibody from each sample of ascites fluid was purified by ammonium sulfate precipitation, anion exchange, cation exchange and affinity chromatography.

Discussion

For the first time, twisted α-keto amides have been used as haptens for the production of monoclonal antibodies to study their potential catalytic properties in acyl-transfer reactions. We conceived the design of these haptens based on experimental findings of the T-cell inhibitors FK 506, rapamycin and the serine protease inhibitors cyclotheonamide A and B.[21,24,48,49]

Structurally the roots of hapten **1** can be traced back to the cyclic peptides cyclotheonamide A and B. It has been

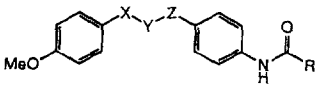
suggested that the α-keto carbonyl group of these peptides may function as an electrophilic mimic of the ArgX scissile amide bond of thrombin substrates.[24] In fact a study by Maryanoff *et al.* demonstrated the inhibition of human α-thrombin by cyclotheonamide A.[48] Furthermore, from X-ray analysis, it was determined that this ligand when complexed to thrombin adopts a conformation which places the two carbonyl groups at a dihedral angle of 109 °. In addition, this study provided insight into the complex hydrogen bonding network which surrounds the α-keto amide.[48] Overall, these intermolecular interactions provide an excellent guide as to possible structural motifs which the antibodies may adopt.

In a similar scenario, X-ray crystallographic studies of immunosuppressant ligands FK 506 and rapamycin have clearly demonstrated that the keto amide carbonyls of the α-keto amides are oriented orthogonally to one another, here the dihedral angle is 95 degrees.[22] Moreover some recent studies have postulated that the α-keto amide of FK 506 and rapamycin is a surrogate for the twisted amide of a peptide substrate.[23] In addition FKBP is classified as a peptidyl-prolyl-*cis-trans* isomerase (rotamase), and it has been shown to catalyze the isomerization of the *cis* amide bond found in proteins and peptide substrates.[50,51] Taken together, these findings suggest that the α-keto amide substructures found in FK 506 and rapamycin mimic a twisted amide bond (i.e. one possible transition-state for amide hydrolysis). In analogy, we believe hapten **2** will adopt orthogonal conformation, which in essence serves as our transition-state analogue for the acyl-transfer reaction. Thus immunogen **2** could elicit antibodies which twist potential substrates into a much more reactive conformation.

There has been a paucity of studies on the α-keto amide functionality, often of great interest is the oxidation state of the α-keto carbonyl. We too were quite interested in the possible hydration state which our hapten could adopt, as this could bias the immune response. Consequently, we have performed ^{13}C NMR studies of haptens **1** and **2**. The ^{13}C NMR spectra were recorded in 10 % D_2O in DMSO-d_6 or DMF-d_7 using 1,4-dioxane as an internal standard.[39] As hoped neither hapten showed any tendency to exist in the hydrated form. We attribute these results to the electron-donating ability of the methoxy substituent found on both rings of **1** and **2**. We speculate that this strongly electron-donating group provides enhanced electron density to the α-keto carbonyl, thereby retarding any chance of hydration. While we cannot predict the immune response, these results provide a more channeled picture as to what types of antibody–antigen interactions we might encounter.

The haptens **1** and **2** which we have profiled offer attractive possibilities for the generation of catalytic antibodies. Both feature an α-keto amide moiety, the so called transition-state functionality, and a *para*-methoxyphenyl appendage this for recognition and immunogenicity. While similar, one key structural attribute differentiates these two haptens, the *N*-methylation of the α-keto amide (i.e. hapten **2**). Such a substitution pattern provides us with a possible window to assess the relevance of the dihedral angle which is observed in FK 506 and rapamycin, and that of cyclotheonamide A.

Overall, we have shown that haptens **1** and **2** are stable haptenic conjugates, which can induce an immune response. We are currently purifying monoclonal antibodies to both structures and we plan to test their potential as credible catalysts. Because of the unknown 'regioselectivity' of these antibodies, we are synthesizing a number of possible substrates (see Figure 3). The results of our kinetic investigations will be published in due course.



ENTRY	X	Y	Z	R
a	C=O	NH	CH_2	$(CH_2)_3CO_2H$
b	C=O	O	CH_2	CH_3
c	O	C=O	CH_2	$(CH_2)_3CO_2H$
d	NH	C=O	CH_2	$(CH_2)_3CO_2H$
e	CH_2	C=O	NH	$(CH_2)_3CO_2H$
f	CH_2	C=O	O	$(CH_2)_3CO_2H$
g	C=O	-	NH	$(CH_2)_3CO_2H$
h	C=O	-	O	$(CH_2)_3CO_2H$
i	O	-	C=O	CH_3
j	NH	-	C=O	$(CH_2)_3CO_2H$
k	C=O	NH	CH_2	CH_3

Figure 3. Structures of the substrates for antibody-catalyzed hydrolytic reactions.

Experimental Section

General methods

Dichloromethane and chloroform were continuously distilled from calcium hydride and P_2O_5, respectively. Tetrahydrofuran (THF) was distilled from sodium metal/benzophenone ketyl. All reagents were purchased from Aldrich Chemical Company. All reactions were carried out under a nitrogen atmosphere with anhydrous solvents under anhydrous conditions, unless otherwise stated. Reactions were monitored by thin-layer chromatography (TLC) using silica gel 60F glass plates (0.25 mm, E. Merck, Darmstadt, Germany) using UV light, 5 % ethanolic phosphomolybdic acid, 1 % ethanolic ninhydrin or *p*-anisaldehyde solution and heat as a developing agent. Flash chromatography was performed with the use of silica gel 60 (230–400 mesh, E. Merck, Darmstadt, Germany) as described by Still.[52] Yields are for unoptimized procedures and refer to chromatographically and spectroscopically (^1H NMR) homogeneous materials, unless otherwise noted.

Melting points are uncorrected and were determined on a Fisher-Johns melting point apparatus. All proton NMR

spectra (300 MHz) were obtained in $CDCl_3$, DMF-d_7 or DMSO-d_6 solutions at ambient temperature on a Bruker AM-300 spectrometer. ^{13}C NMR spectra (500 MHz) were recorded on a Bruker AMX-500 instrument. Chemical shifts (δ) are reported in parts per million downfield from tetramethylsilane (TMS) as an internal reference at 0.00 ppm. Coupling constants are reported in Hertz. Low resolution liquid secondary ion mass spectra were provided by Dr Gary Siudzak of The Scripps Research Institute Mass Spectrometry Facility.

Synthesis of the hapten 1

5-[(4-Nitrophenyl)amino]-5-oxopentanoic acid (3). A mixture of 4-nitroaniline (4.00 g, 29.0 mmol) and glutaric anhydride (3.30 g, 29.0 mmol) in $CHCl_3$ (120 mL) was refluxed for 3 h. The reaction mixture was filtered and the product washed with EtOAc and Et_2O. Recrystallization from EtOH gave **3** (7.02 g, 96 %): mp 169–170 °C (lit.[23] 170–171 °C); ^1H NMR (DMSO-d_6) δ 10.43 (br s, 1 H), 8.19 (d, J = 9 Hz, 2 H), 7.79 (d, J = 9 Hz, 2 H), 2.40 (t, J = 7 Hz, 2 H), 2.25 (t, J = 7 Hz, 2 H), 1.79 (qn, J = 7 Hz, 2 H); LSIMS$^+$ 253 (M + H$^+$).

Methyl 5-[(4-nitrophenyl)amino]-5-oxopentanoate (4). A solution of **3** (6.00 g, 23.8 mmol) and concentrated sulfuric acid (0.2 mL) in MeOH (150 mL) was refluxed for 2 h. The product crystallized on cooling and was washed with cold MeOH and Et_2O to afford **4** (6.08 g, 96 %): ^1H NMR (DMSO-d_6) δ 11.22 (br s, 1 H), 8.16 (d, J = 8 Hz), 7.79 (d, J = 8 Hz, 2 H), 3.56 (s, 3 H), 2.25–2.45 (t + t, J = 7 Hz, 4 H), 1.82 (qn, J = 7 Hz, 2 H); LSIMS$^+$ 267 (M + H$^+$).

Methyl 5-[(4-aminophenyl)amino]-5-oxopentanoate (5). To a mixture of **4** (6.00 g, 22.5 mmol) in MeOH (80 mL) was added 10 % palladium on activated carbon (0.75 g). The reaction vessel was pressurized to 45 psi with H_2 on a Parr apparatus for 1.5 h. The mixture was filtered through Celite and evaporated to dryness. The crude product was purified by flash chromatography using EtOAc–TEA (20:1), yielding 4.93 g (93 %) of **5**: ^1H NMR (DMSO-d_6) δ 9.43 (br s, 1 H), 7.14 (d, J = 8 Hz, 2 H), 6.45 (d, J = 8 Hz, 2 H), 3.56 (s, 3 H), 2.31 (t, J = 7 Hz, 2 H), 2.22 (t, J = 7 Hz, 2 H), 1.78 (qn, J = 7 Hz, 2 H); LSIMS$^+$ 237 (M + H$^+$).

4-Methoxyphenylglyoxylic acid (6). (±)-4-Methoxymandelic acid (5.00 g, 27.5 mmol) was dissolved in 0.40 M NaOH solution (145 mL, 58.0 mmol) in H_2O. The mixture was cooled to 0 °C and $KMnO_4$ (3.01 g, 19.0 mmol) was added in small portions over a period of 30 min. The reaction mixture was stirred at 0 °C for 4 h. Then, EtOH (50 mL) was added, and the mixture stirred at room temperature for 45 min. The mixture was filtered through Celite, acidified with concentrated hydrochloric acid and extracted with EtOAc. The combined organic layers were washed with brine, dried with $MgSO_4$, filtered and evaporated *in vacuo*. The crude product was chromatographed on silica, eluting with EtOAc–hexane (4:1) to give **6** (4.10 g, 83 %): ^1H NMR (DMSO-d_6) δ 7.88 (d, J = 10 Hz, 2 H), 7.12 (d, J = 10 Hz, 2 H); LSIMS$^+$ 181 (M + H$^+$).

Methyl 5-[[4-[[(4-methoxyphenyl)oxoacetyl]amino]-phenyl]amino]-5-oxopentanoate (7). A solution of **6** (0.11 g, 0.62 mmol), **5** (0.15 g, 0.62 mmol) and TEA (0.17 mL, 1.23 mmol) in CH_2Cl_2 (4 mL) was stirred at 0 °C for 3 min. Then, BOP-Cl (0.16 g, 0.62 mmol) was added and the reaction mixture was stirred at 0 °C for 1 h. The mixture was acidified with 1 M HCl (10 mL), and the precipitate was filtered, washed with H_2O (3 x 5 mL) and Et_2O (3 x 5 mL) and dried to provide **7** (0.21 g, 85 %): ^1H NMR (DMSO-d_6) δ 10.01 (br s, 1 H), 9.83 (br s, 1 H), 7.92 (d, J = 10 Hz, 2 H), 7.63 (d, J = 9 Hz, 2 H), 7.52 (d, J = 9 Hz, 2 H), 7.03 (d, J = 10 Hz, 2 H), 3.82 (s, 3 H), 3.58 (s, 3 H), 2.27–2.39 (obsc., 4 H), 1.80 (qn, J = 7 Hz, 2 H); LSIMS$^+$ 503 (M + Cs$^+$ - CO).

5-[[4-[[(4-Methoxyphenyl)oxoacetyl]amino]phenyl]-amino]-5-oxopentanoic acid (1). A mixture of **7** (0.10 g, 0.25 mmol) and NaOH (0.10 g, 2.51 mmol) in H_2O (4 mL) was stirred at room temperature for 20 min. 1 M HCl (10 mL) was added and the precipitate was filtered and washed with H_2O (2 x 5 mL) and Et_2O (2 x 5 mL). Column chromatography (PhMe–EtOAc–HOAc 5:5:1) gave **1** (88 mg, 92 %): ^1H NMR (DMSO-d_6) δ 9.99 (br s, 1 H), 9.82 (br s, 1 H), 7.90 (d, J = 10 Hz, 2 H), 7.63 (d, J = 9 Hz, 2 H), 7.49 (d, J = 9 Hz, 2 H), 7.01 (d, J = 10 Hz, 2 H), 3.80 (s, 3 H), 2.18–2.37 (obsc., 4 H), 1.78 (qn, J = 7 Hz, 2 H); ^{13}C NMR (DMF-d_7) δ 173.9, 170.4, 164.6, 162.1, 135.3, 134.9, 129.2, 127.2, 120.3, 119.0, 113.3, 54.9, 35.4, 32.8, 20.6; LSIMS$^+$ 357 (M + H$^+$ - CO).

Synthesis of the hapten 2

Methyl 5-[[4-[[(1,1-dimethylethoxy)carbonyl]amino]-phenyl]amino]-5-oxopentanoate (8). A mixture of **5** (5.25 g, 22.2 mmol) and di-*tert*-butyl dicarbonate (4.85 g, 22.2 mmol) in 1,4-dioxane (40 mL) was stirred at 85 °C for 12 h. The solvent was removed *in vacuo*, and the residue was chromatographed on silica (EtOAc) to give pure **8** (6.08 g, 81 %): ^1H NMR (DMSO-d_6) δ 9.76 (br s, 1 H), 9.20 (br s, 1 H), 7.51 (d, J = 8 Hz, J = 8 Hz, 2 H), 7.29 (d, J = 8 Hz, 2 H), 3.55 (s, 3 H), 2.22–2.48 (obsc. 4 H), 1.79 (qn, J = 7 Hz, 2 H), 1.43 (s, 9 H); LSIMS$^+$ 359 (M + Na$^+$), 337 (M + H$^+$).

Methyl 5-[[4-[[(1,1-dimethylethoxy)carbonyl]methyl-amino]phenyl]methylamino]-5-oxopentanoate (9). Iodomethane (1.14 mL, 11.9 mmol) was added dropwise through a septum to a stirred mixture of **8** (2.00 g, 5.95 mmol) and potassium *tert*-butoxide (1.34 g, 11.9 mmol) in THF (20 mL). The reaction mixture was stirred at room temperature for 45 min. The mixture was filtered and evaporated *in vacuo*. The oily residue was chromatographed on silica (MeCN–CH_2Cl_2 1:2) to provide **9** (1.96 g, 90 %): ^1H NMR (DMSO-d_6) δ 7.31 (d, J = 8 Hz, 2 H), 7.23 (d, J = 8 Hz, 2 H), 3.49 (s, 3 H), 3.14 (br s, 3 H), 3.08 (br s, 3 H), 2.19 (br t, 2 H), 1.98 (br t, 2 H), 1.67 (qn, J = 7 Hz, 2 H), 1.38 (s, 9 H); LSIMS$^+$ 497 (M + Cs$^+$).

Methyl 5-[methyl[4-(methylamino)phenyl]amino]-5-oxopentanoate (10). A mixture of **9** (1.20 g, 3.29 mmol) and trifluoroacetic acid (2.5 mL, 32.9 mmol) in CH_2Cl_2 (10 mL) was stirred at ambient temperature for 25 min. The volatiles were removed *in vacuo*, and the residue was dissolved in CH_2Cl_2. TEA (0.7 mL, 5.0 mmol) was added to the mixture and the solution was washed with brine. CH_2Cl_2 was dried with $MgSO_4$, filtered and evaporated to dryness to give **10** (0.66 g, 76 %): ^{1}H NMR (DMSO-d_6) δ 6.93 (d, J = 8 Hz, 2 H), 6.49 (d, J = 8 Hz, 2 H), 5.82 (q, J = 3 Hz, 1 H), 3.50 (s, 3 H), 3.03 (s, 3 H), 2.63 (d, J = 3 Hz, 3 H), 2.18 (t, J = 7 Hz, 2 H), 1.96 (t, J = 7 Hz, 2 H), 1.61 (qn, J = 7 Hz, 2 H); LSIMS$^+$ 397 (M + Cs$^+$).

Methyl 5-[[4-[[(4-methoxyphenyl)oxoacetyl]methylamino]phenyl]methylamino]-5-oxopentanoate (11). BOP-Cl (0.17 g, 0.66 mmol) was added to a stirred mixture of **6** (0.12 g, 0.66 mmol), **10** (0.18 g, 0.66 mmol) and TEA (0.19 mL, 1.33 mmol) in CH_2Cl_2 (2 mL). The reaction mixture was stirred at room temperature for 36 h. The mixture was acidified with 1 M HCl (10 mL) and extracted with CH_2Cl_2. The combined organic layers were washed with brine, dried with $MgSO_4$, filtered and solvent removed *in vacuo*. The residue was chromatographed on silica (EtOAc–hexane 3:1) to afford **11** (0.26 g, 92 %): ^{1}H NMR (DMSO-d_6) δ 7.13–7.24 (obsc., 6 H), 6.73 (d, J = 8 Hz, 2 H), 3.68 (s, 3 H), 3.51 (s, 3 H), 3.39 (s, 3 H), 3.09 (br s, 3 H), 2.18 (br t, 2 H), 1.90 (br t, 2 H), 1.63 (qn, J = 7 Hz, 2 H); LSIMS$^+$ 399 (M + H$^+$ -CO), 531 (M + Cs$^+$ - CO).

5-[[4-[[(4-Methoxyphenyl) oxoacetyl] methylamino]-phenyl]-methylamino]-5-oxopentanoic acid (2). A mixture of **11** (86 mg, 0.20 mmol) and NaOH (81 mg, 2.02 mmol) in H_2O (1 mL) was stirred at 0 °C for 20 h. The mixture was acidified with 1 M HCl (10 mL) and extracted with EtOAc. The combined EtOAc layers were washed with brine, dried with $MgSO_4$, filtered and evaporated *in vacuo*. The residue was chromatographed on silica (PhMe–EtOAc–HOAc 5:5:1) to provide **2** (63 mg, 76 %): ^{1}H NMR ($CDCl_3$) δ 7.39 (d, J = 8 Hz, 2 H), 7.02–7.16 (obsc., 4 H), 6.69 (d, J = 8 Hz, 2 H), 3.74 (s, 3 H), 3.49 (s, 3 H), 3.18 (br s, 3 H), 2.25 (br t, 2 H), 1.98 (br t, 2 H), 1.82 (br qn, 2 H); ^{13}C NMR (DMSO-d_6) δ 189.5, 174.0, 166.6, 164.1, 142.9, 131.9, 131.5, 127.8, 125.6, 114.8, 114.3, 55.7, 35.4, 32.7, 32.4, 32.2, 20.1; LSIMS$^+$ 407 (M + Na$^+$ - CO).

Preparation of the immunoconjugates

The haptens **1** and **2** were activated by adding 1.3 mole equivalents of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and *N*-hydroxysulfosuccinimide aqueous solutions to a solution of haptens **1** and **2** (4 mg) in *N,N*-dimethylformamide (200 µl). Activation reactions were incubated at room temperature for 24 h.

The keyhole limpet hemocyanin (KLH) conjugate was prepared by adding 100 µl of the activated hapten solution to a solution of 5 mg of KLH in 900 µl of 50 mM sodium phosphate buffer (pH 7.5). The bovine serum albumin (BSA) conjugate was prepared in a similar fashion. The hapten–protein conjugates were incubated at 4 °C for 24 h. The protein conjugates were used as such in immunization without further purification.

Immunization and production of monoclonal antibodies

Two intraperitoneal (ip) injections of RIBI adjuvant (MPL and TDM emulsions) and 100 µg of the hapten conjugated to KLH were administered to four 8-week-old 129GIX$^+$ mice two weeks apart. One month after the second injection, the mouse with the highest titer (12,800 to 25,600) was injected intravenously (iv) with 50 µg of KLH conjugate;[46,47] 3 days later, the spleen was taken for the preparation of hybridoma cells. Spleen cells (1.0 x 10^8) were fused with SP2/0 myeloma cells (2.0 x 10^7). Cells were plated into 30, 96-well plates; each well contained 150 µl of hypoxanthine, aminopterin, thymidine–Dulbecco's minimal essential medium (HAT–DMEM) containing 1 % Nutridoma® and 2 % bovine serum albumin.

After 2 weeks, the antibodies produced in the wells containing macroscopic colonies were assayed by ELISA for binding to **1** and **2**, respectively. The colonies that initially produced antibodies, which bound **1** and **2**, respectively, were subcloned twice, after which 22 mAbs for the hapten **1** and 26 for the hapten **2** remained active. The subtype distribution of the 22 monoclonal antibodies was 6 immunoglobulin G1 (IgG1), 11 $IgG2_a$ and 5 $IgG2_b$ for hapten **1**. For the hapten **2**, the subtype distribution of the 26 mAbs was 4 IgG1, 16 $IgG2_a$, 5 $IgG2_b$ and 1 IgG3. All monoclonal antibodies were injected into pristane[53]-primed 129GIX$^+$ × BALB/C mice to generate ascites.[54]

The globular fractions from ascites were precipitated by dropwise addition of saturated ammonium sulfate at 4 °C, pH 7.2, to achieve a final concentration of 45 %. Ammonium sulfate was removed by dialysis against 10 mM Tris, pH 8. The concentrated antibodies were next purified by anion exchange chromatography on DEAE-Sephacel® and eluted with a stepwise NaCl gradient (50 to 500 mM NaCl). The antibodies eluted in the 50–100 mM NaCl fraction and were concentrated by ultrafiltration prior to cation exchange chromatography on a mono Q column. The antibody was loaded on the column, and nonadherent material removed by extensive washings (50 mM Tris, pH 7.8). The column was eluted with a stepwise NaCl gradient (0 to 500 mM NaCl, 50 mM Tris, pH 8.5). The antibody eluted was again concentrated by ultrafiltration. This material was then affinity purified on a protein G-Sepharose column. In all cases antibody was loaded onto the column, and nonadherent material was removed by extensive washings (20 to 30 column volumes). The column was eluted with 0.05 M citric acid, pH 3.0, and fractions were immediately neutralized by collecting into 1 M Tris, pH 9.0.

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