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Design and Syntheses of Three Haptens to Generate Catalytic Antibodies that Cleave Amide Bonds with Nucleophilic Catalysis

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Abstract—Design principles and syntheses of three haptens that were recently reported to generate amide bond cleaving catalytic antibodies are described. The hapten designs sought to induce acidic and/or basic residues in antibody binding sites via charge complementarity, and also to generate a hydrophobic binding pocket for an external phenol nucleophile. The charged yet aromatic nature of these haptens presented some unique synthetic challenges and solutions to which are described below.

Introduction

In our recent work, we have focused on the generation of antibody catalysts for the cleavage of amide bonds,[1] a highly challenging task.[2] To this end, we devised a heterologous immunization protocol where two structurally similar but differently functionalized haptens are used to immunize animals in sequence. The aim of this approach is to generate multiple charged residues in antibody binding sites while avoiding complicated syntheses of zwitterionic haptens. This method yielded highly efficient esterolytic antibodies, although amide hydrolysis met with little success.

A novel approach to amide hydrolysis was adopted in the present work with the use of an externally supplied nucleophilic cofactor.[3] This approach would pattern after the mechanism of serine proteases,[4] which use an active-site serine residue to initiate the nucleophilic attack on the peptide bond. We chose phenol as the externally supplied nucleophile for antibody catalyzed cleavage of the amide bond which would generate a water labile phenyl ester. In addition to the high water solubility and good nucleophilicity of phenol, its aromatic ring provides a convenient recognition element for antibody binding pockets via hydrophobic interactions. Propionyl *p*-nitroanilide was chosen as a simple chromogenic amide substrate. This mechanism is shown in Scheme 1.

A unimolecular version of this reaction was also designed where the phenolic group is tethered to the amide as shown in substrate **1** (Fig. 1). This substrate technically simplified the initial screening where 74 different clones were tested. Three catalysts were found to accelerate the intramolecular reaction.[3a] When these clones were tested in the presence of phenol and propionyl *p*-nitroanilide, all three were found to accelerate this target amide cleavage reaction, although, of course, with kinetic constants different from those obtained in the unimolecular case.[3b]

In both reaction systems, antibody 14–10 generated by heterologous immunization was found to be a fivefold to tenfold more efficient catalyst (in terms of k_{cat}/K_m) than the antibodies 6–17 and 3–49 raised by homologous immunization with only one of the haptens. Furthermore, the percentage of catalysts among all hapten binding antibodies was significantly higher with the heterologous immunization protocol (ca. 20%) than with either one of the homologous immunization protocols (ca. 5%).

The three haptens (**O1, O2, O3**) were designed to generate antibody binding pockets with the following important features: (1) a hydrophobic binding pocket for the phenyl ring, (2) an acidic residue complementary to the oxyanionic transition state (**O1, O3**), and (3) a basic residue to aid the deprotonation of the phenol nucleophile and hopefully protonation of the departing amine afterwards (**O2**). In addition to homologous immunization using each of the haptens separately, hapten **O2** would be paired with either **O1** or **O3** in a heterologous immunization protocol. Upon initial immunizations, *N*-oxide hapten **O3** failed to generate a strong immune response and therefore, all further studies

Key words: Antibodies; catalysts; phosphonamidates; quinolizinium ions.

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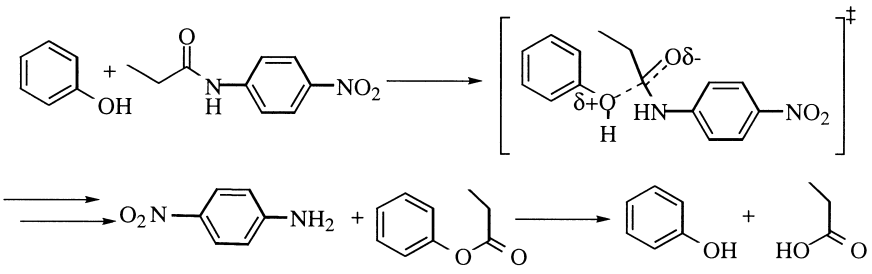


Scheme 1.

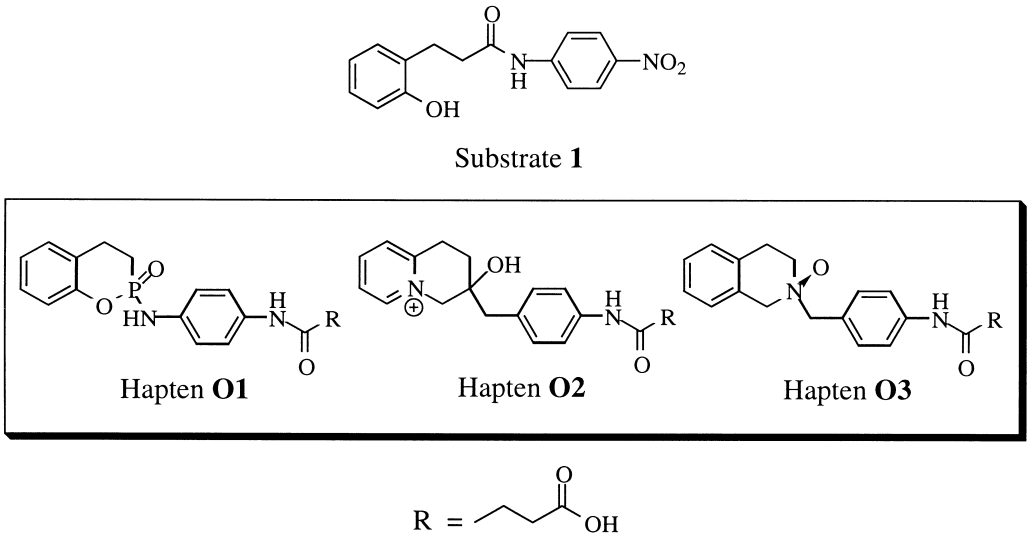


Figure 1.

were carried out with haptens **O1** and **O2**. Since kinetic studies on the antibody catalysis were discussed extensively,[3] this article describes the syntheses of haptens **O1, O2** and **O3** to complete a full documentation of this subject.

Results and discussion

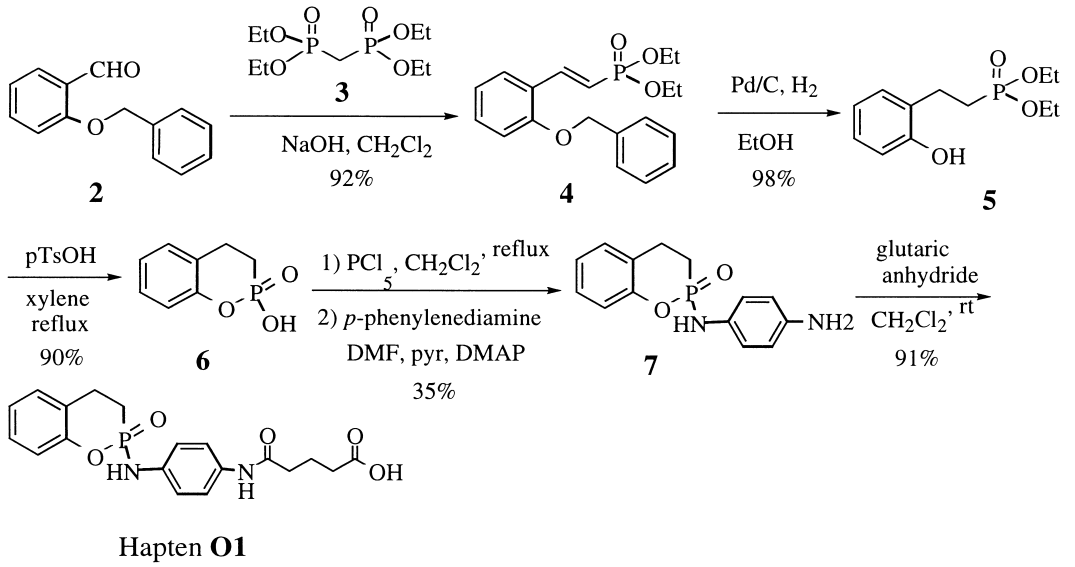
The synthesis of hapten **O1** (Scheme 2) started with 2-benzyloxybenzaldehyde **2**, easily prepared by benzylation of commercially available salicylaldehyde. The Horner–Emmons olefination of **2** with bisphosphonate **3** under biphasic conditions[5] provided *trans*-stilbenephosphonate **4** in good yield. Catalytic hydrogenation of **4** reduced the olefin and removed the benzyl protecting group in one step, which yielded diethyl phosphonate **5**.

Since the phosphonamidate functionality present in hapten **O1** is known to be rather labile,[6] it was decided to first construct a new P–O bond (to cyclize **5**) rather than a P–N bond. Thus phosphonate **5** was treated with PCl_5 in order to carry out the cyclization step. Although one of the ethoxy groups of the phosphonate **5** was easily exchanged for chloride, various attempts failed to yield the desired phosphonic acid **6**. Other strategies such as generating the monophosphonic acid and treating it with various acyl-coupling reagents (e.g. BOP[7] or carbodiimides) were also unsuccessful.[8] Finally, treatment of **5** with anhydrous *p*-toluenesulfonic acid (in refluxing xylene to azeotropically remove generated ethanol), afforded the cyclic phosphonate **6**, achieving two steps in a one-pot reaction in good yield (90%).

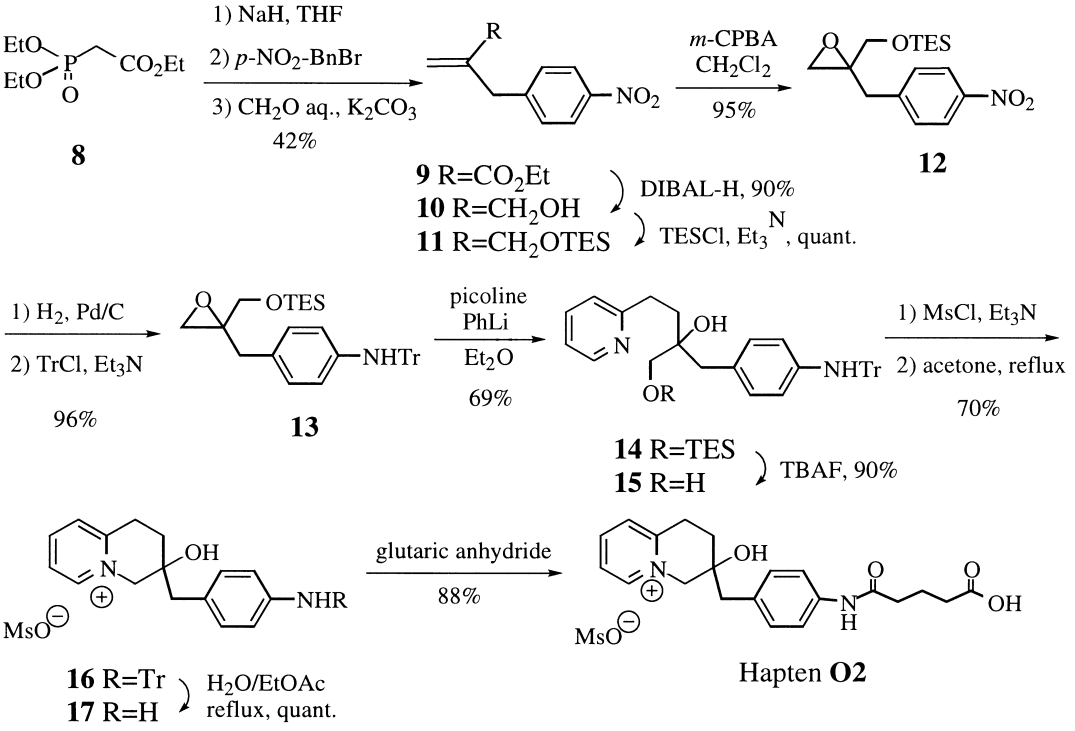
The formation of the phosphonamidate bond was not straightforward. The phosphonyl chloride of **6** was found to be very unreactive and failed to react with 4-nitroaniline, aniline or even with a carbamate protected phenylenediamine under various reaction conditions.[9] Only unprotected phenylenediamine (in excess) showed the required nucleophilicity to react with this phosphonyl chloride. The problem of handling excess of phenylenediamine was solved by a careful and exhaustive purification which involved removal of the excess of phenylenediamine via sublimation, followed by two flash column chromatography steps to afford the desired phosphonamidate **7**. This compound was treated with glutaric anhydride to give hapten **O1** (5 steps, 26% overall yield).

The synthesis of hapten **O2** was accomplished in 13 steps (10% overall yield) as shown in Scheme 3. Alkylation of the phosphonate **8** with 4-nitrobenzyl bromide[10] followed by a Horner–Emmons reaction with formaldehyde[11] afforded the unsaturated ester **9**.

The DIBAL-H reduction of ester **9** at −78 °C, followed by triethylsilyl protection of the resulting primary alcohol



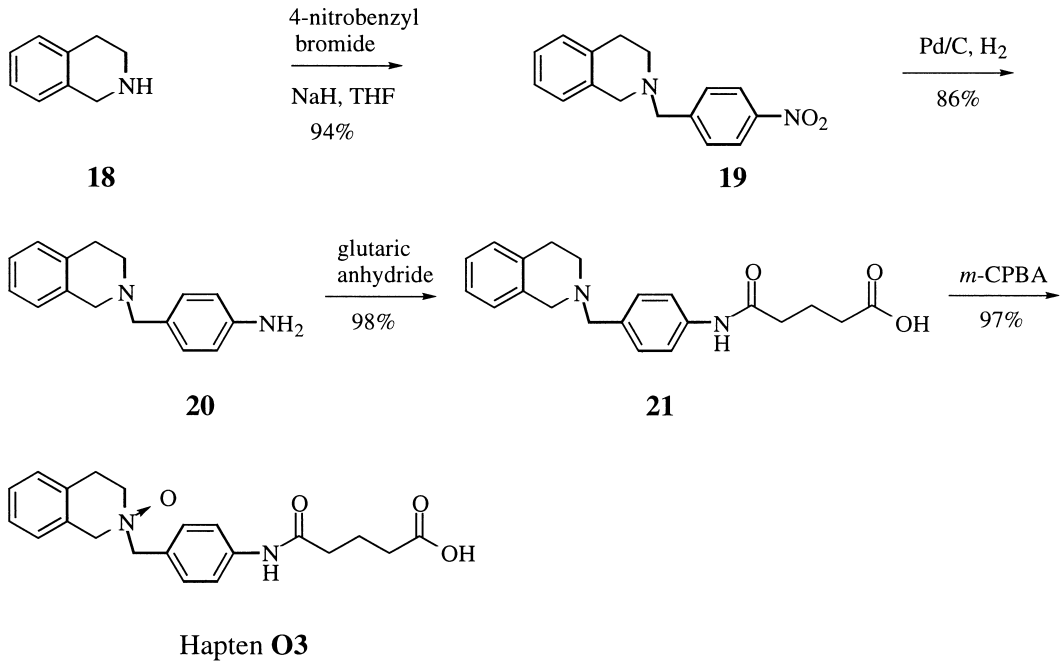
Scheme 2.



Scheme 3.

10 and subsequent epoxidation of the double bond with *m*-CPBA provided **12** in good yield (86%, 3 steps). The nitro group of **12** impeded the nucleophilic opening of the epoxide with 2-picolyllithium even when high order cyano cuprates[12] were used, so we decided to reduce it to an amine and protect it. Hydrogenation of the nitro group with Pd/C followed by protection of the resulting amino moiety with trityl chloride proceeded almost quantitatively. The opening of the epoxide **13** with 2-picolyllithium[13] proceeded then smoothly to give **14** in 69% yield. Deprotection of the TES group with TBAF gave the diol **15**. Activation of the primary hydroxyl group with methanesulfonyl chloride (MsCl) and subsequent cyclization in refluxing acetone[14] afforded the quinolizinium salt, **16**. Finally, the trityl-group of **16** was removed under biphasic conditions by refluxing it in a mixture of water/ethyl acetate, followed by condensation of obtained **17** with glutaric anhydride to yield hapten **O2** as an amorphous solid.

The synthesis of hapten **O3** was straightforward as shown in Scheme 4. Thus, 1,2,3,4 tetrahydro-isoquinoline **18** was alkylated with 4-nitrobenzylbromide to give **19** followed by the catalytic reduction of the nitro



Scheme 4.

group. The thus obtained aniline **20** was coupled with glutaric anhydride to afford **21**. The tertiary amine **21** was relatively unreactive towards oxidation to the *N*-oxide, and after several attempts (including different peroxides and dioxirane) *m*-CPBA was found to be the most efficient reagent providing hapten **O3** in 77% overall yield (4 steps).

In summary, we have disclosed here the full experimental details of the syntheses of three novel haptens that were used to generate amide cleaving antibodies. Considerable synthetic challenges were encountered especially in the synthesis of hapten **O1** both in the cyclization of phosphonate **5**, and also in the formation of the P–N bond of phosphonamidate **7**. A novel acid catalyzed cyclization protocol followed by a careful matching of phosphonyl and amine reactivities helped to successfully overcome the respective challenges.

Experimental

Air sensitive reactions were carried out under an argon atmosphere in oven-dried glassware. Flash chromatography was performed using Merck silica gel 60 (230–400 mesh). Solvents used for elution are noted for the specific examples. Solvents extracts were routinely dried over $MgSO_4$ and concentrated. Anhydrous solvents, starting materials, and reagents were purchased from Aldrich Chemical Co. and used as obtained. THF and Et_2O were distilled from Na/benzophenone. CH_2Cl_2 and pyridine were distilled from CaH_2. Proton (^1H NMR) magnetic resonance were recorded at 300 MHz on a Varian XL-300 instrument. The chemical shifts of ^1H NMR spectra were referenced to $CDCl_3$ (7.24 ppm), DMSO (2.49 ppm), CD_2Cl_2 (5.32 ppm), or CD_3OD (3.30 ppm). ^1H NMR peak multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), c (quartet), q (quintuplet), m (multiplet), br (broad). Coupling constants (*J*) are reported in Hz. Proton-decoupled-carbon (^{13}C NMR) and phosphorus (^{31}P NMR) magnetic resonance spectra were recorded at 75.44 MHz and 121.44 MHz, respectively, on a Varian XL-300 instrument. The chemical shifts of ^{13}C NMR spectra were referenced to $CDCl_3$ (77.0 ppm), DMSO (39.5 ppm), CD_2Cl_2 (53.8 ppm), or CD_3OD (49.0 ppm). ^{31}P NMR spectra were referenced to 85% H_3PO_2 as an external standard (capillary tube within NMR tube). Infrared (IR) spectra were recorded on a Perkin–Elmer 283B spectrometer. Melting points were not corrected. High-resolution mass spectra (HRMS) were recorded on a Finnigan MAT 8200 spectrometer.

2-Benzyloxybenzaldehyde (2). A solution of anhydrous K_2CO_3 (29.9 g, 216 mmol) in MeOH (300 mL) and $CHCl_3$ (600 mL) was refluxed for 15 min under a stream of argon. Salicylaldehyde (5.25 mL, 49.1 mmol) and benzyl bromide (6.45 mL, 54.0 mmol) were added, and the resulting mixture was refluxed for 1 day. The reaction mixture was filtered, the filtrate evaporated and the resulting residue was partitioned between 1N HCl (200 mL) and CH_2Cl_2 (2×300 mL). The combined organic layers were washed with brine, dried, filtered and concentrated to afford 10.3 g (99%) of **2** as a slightly yellow oil. ^1H NMR of the crude showed it to be very pure and it was used in the next step without any further purification. IR (film) 3053, 3020, 2751, 1687, 1598 cm^{-1}. ^1H NMR ($CDCl_3$) δ 10.57 (s, 1H), 7.86 (d, $J=8.0$ Hz, 1H), 7.57–7.49 (m, 1H), 7.47–7.32 (m, 5H), 7.08–7.00 (m, 2H), 5.19 (s, 2H).

(*E*) Diethyl [(2-benzyloxy)phenyl]-ethenyl-phosphonate (4). NaOH (50% aq, 6 mL) was added to a solution of **2** (0.74 g, 3.48 mmol) and bisphosphonate **3** (0.86 mL,

3.48 mmol) in CH_2Cl_2 (6 mL). The biphasic reaction was stirred vigorously (open to ambient athmosphere) for 4 h. The reaction mixture was diluted with H_2O (20 mL) and extracted with CH_2Cl_2 (2×20 mL). The combined organic layers were washed with brine, dried, filtered and concentrated to give 1.1 g (92%) of **4** as a colorless oil. IR (film) 3209, 2977, 2745, 2612, 1594, 1500, 1456 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.88 (dd, $J_{P-H}=23.4$ Hz, $J_{H-H}=17.7$ Hz, 1H), 7.46 (dd, $J=4.8$, 1.5 Hz, 1H), 7.41–7.21 (m, 6H), 6.92 (t, $J=9.0$ Hz, 2H), 6.32 (dd, $J_{P-H}=19.5$ Hz, $J_{H-H}=17.7$ Hz, 1H), 5.19 (s, 2H), 4.07 (q, $J=7.2$ Hz, 4H), 1.28 (t, $J=7.2$ Hz, 6H); ^{13}C NMR ($CDCl_3$) δ 154.3, 143.9 (d, $J_{C-P}=117.2$ Hz), 141.2 (d, $J_{C-P}=11.1$ Hz), 133.5, 131.7, 128.7, 128.5, 128.3, 127.6, 127.0, 126.6, 122.8, 63.3, 62.1 (d, $J_{C-P}=4.9$ Hz), 31.8. HRMS (M^+) for $C_{19}H_{23}O_4P$: calcd. 346.3630, found 346.3629.

Diethyl 2-(2-hydroxyphenyl)-ethyl-phosphonate (5). Pd/C (10%, catalytic amount) was added to a solution of **4** (1.99 g, 5.72 mmol) in deoxygenated EtOH (30 mL). The flask was purged with argon and then evacuated (vacuum) and pressurized (H_2) three times. The reaction mixture was mechanically shaken under 52 psi of H_2 for 3 days. The catalyst was removed by filtration, and the filtrate was evaporated to afford 1.45 g (98%) of **5** as a colorless oil. The crude product was used in the next step without further purification: ^1H NMR ($CDCl_3$) δ 8.45–8.10 (br, 1H), 7.10–7.02 (m, 2H), 6.88 (d, $J=8.2$ Hz, 2H), 6.78 (t, $J=7.8$ Hz, 2H), 3.99 (q, $J=7.2$ Hz, 4H), 2.89 (dt, $J_{P-H}=22.8$ Hz, $J_{H-H}=7.2$ Hz, 1H), 2.12 (dt, $J_{P-H}=17.1$ Hz, $J_{H-H}=7.2$ Hz, 1H), 1.33 (t, $J=7.2$ Hz, 6H). ^{13}C NMR ($CDCl_3$) δ 131.7, 130.8, 128.6, 128.2, 126.6, 121.5, 62.1 (d, $J_{C-P}=4.8$ Hz), 31.8, 25.6 (d, $J_{C-P}=11.5$ Hz), 20.5 (d, $J_{C-P}=125.6$ Hz). ^{31}P NMR ($CDCl_3$) δ 45.58. HRMS (M^+) for $C_{12}H_{19}O_4P$: calcd. 258.2542, Found 258.2537.

2-Hydroxy-3,4-dihydro-1,2-benzoxaphosphorin-2-oxide (6). A solution of **5** (1.8 g, 6.97 mmol) and anhydrous *p*-toluenesulfonic acid (1.57 g, 9.07 mmol) in xylene (40 mL) were refluxed open to the air for 24 h. The reaction mixture was cooled to rt, concentrated in vacuo to a volume of 3–4 mL and stored at 5 °C overnight. The resulting white solid was filtrated and recrystallized from acetone to give 1.15 g (90%) of **6**. ^1H NMR (DMSO-d_6) δ 7.29–7.16 (m, 2H), 7.12–6.98 (m, 2H), 3.04 (dt, $J_{P-H}=23.6$ Hz, $J_{H-H}=7.4$ Hz, 2H), 2.12 (dt, $J_{P-H}=17.9$ Hz, $J_{H-H}=7.4$ Hz, 2H). ^{13}C NMR (DMSO-d_6) δ 129.8, 129.5, 128.7, 127.7, 124.9, 119.3 (d, $J_{C-P}=7.5$ Hz), 32.5 (d, $J_{C-P}=9.8$ Hz), 28.0 (d, $J_{C-P}=107.9$ Hz). ^{31}P NMR (DMSO): δ 25.76. HRMS (M^+) for $C_8H_9O_3P$: calcd. 184.1314, Found 184.1311.

2-[(4-Aminophenyl)amino]-3,4-dihydro-1,2-benzoxaphosphorin-2-oxide (7). A suspension of **6** (1.15 g, 6.3 mmol) and PCl_5 (2.0 g, 9.6 mmol) in CH_2Cl_2 (10 mL) was refluxed overnight. Xylene was added and the mixture was concentrated in vacuo. Azeotropic removal of xylene was repeated several times to remove the $POCl_3$ by-product of the reaction. The phosphonyl chloride obtained (0.77 g, 3.8 mmol) was redissolved in CH_2Cl_2 (10 mL) and added dropwise (over 1 h) via an addition-funnel to a solution of freshly-sublimed phenylene diamine (0.87 g, 8.0 mmol), pyridine (0.67 mL, 8.0 mmol) and DMAP (cat.) in DMF (2 mL). The resulting mixture was stirred for 6 h at rt. The reaction mixture was diluted with CH_2Cl_2 (40 mL), filtered (in order to remove the pyridinium chloride) and the filtrate was concentrated. The excess of unreacted phenylene diamine was removed by sublimation in vacuo (1 mm Hg, 120 °C) and the remaining residue was purified by flash chromatography (gradient 2 to 5% MeOH in CH_2Cl_2). The fractions containing the product **7** with the contaminating phenylene diamine were then pooled, concentrated in vacuo, and purified again by flash chromatography (gradient 75–100% EtOAc–hexanes with 0.1% NH_4OH) to yield 364 mg (35%) of **7** as a white solid. This compound is stable indefinitely under ambient atmosphere. ^1H NMR (CD_3OD) δ 7.26–7.12 (m, 2H), 7.08–6.94 (m, 4H), 6.80 (d, $J=8.4$ Hz, 2H), 3.18–3.02 (m, 2 H), 2.27–2.11 (m, 2 H). ^{13}C NMR (DMSO-d_6) δ 150.9 (d, $J_{C-P}=6.6$ Hz), 136.5, 132.6, 128.6, 127.7, 124.2 (d, $J_{C-P}=10.4$ Hz), 123.3, 121.0 (d, $J_{C-P}=5.5$ Hz), 118.3 (d, $J_{C-P}=6.9$ Hz), 118.2, 23.9 (d, $J_{C-P}=9.3$ Hz), 20.5 (d, $J_{C-P}=115.9$ Hz). ^{31}P NMR (DMSO-d_6) δ 27.16. HRMS (M^+) for $C_{14}H_{15}O_2PN_2$: calcd. 274.2591, found 274.2592.

5-({4-[2-(3,4-Dihydro-1,2-benzoxaphosphorin-2-oxide)-amino]phenyl} amino)-5-oxo-pentanoic acid (hapten O1). A mixture of phosphonamidate **7** (215 mg, 0.785 mmol) and glutaric anhydride (990 mg, 0.86 mmol) in CH_2Cl_2 (20 mL) were stirred at rt for 3 h. The precipitated white solid was filtered and dried under vacuum to afford 275 mg (91%) of hapten **O1**, which can be stored indefinitely. ^1H NMR (CD_3OD) δ 7.36 (d, $J=9.6$ Hz, 2H), 7.21–7.13 (m, 2H), 7.04–6.95 (m, 4H), 3.20–3.02 (m, 2H), 2.35 (t, $J=7.5$ Hz, 2H), 2.34 (t, $J=7.5$ Hz, 2H), 2.24–2.11 (m, 2H), 1.94 (q, $J=7.2$ Hz, 2H). ^{13}C NMR (CD_3OD) δ 176.4, 172.8, 152.1 (d, $J_{C-P}=8.0$ Hz), 136.1, 134.0, 129.9, 129.0, 125.3 (d, $J_{C-P}=12.7$ Hz), 124.6, 122.1, 120.3 (d, $J_{C-P}=5.7$ Hz), 119.7 (d, $J_{C-P}=6.9$ Hz), 36.5, 33.8, 25.2 (d, $J_{C-P}=8.1$ Hz), 21.8 (d, $J_{C-P}=114.3$ Hz), 21.6. ^{31}P NMR (CD_3OD) δ 27.49. HRMS (M^+) for $C_{19}H_{21}O_5PN_2$: Calcd. 388.3600, found 388.3598.

Ethyl 2-[(4-nitrophenyl)methyl]-acrylate (9). A solution of triethyl phosphono-acetate **8** (900 μL, 4.5 mmol) in THF (5 mL) was added dropwise to a suspension of sodium hydride (200 mg, 5 mmol, 80%) in THF (5 mL) at 0 °C. The resulting mixture was allowed to stir at rt for 15 min (potassium salt precipitated as white solid) followed by the addition of a solution of 4-nitro-benzylbromide (970 mg, 4.5 mmol) in THF (5 mL). The resulting solution was stirred for 1 h at rt (the precipitate was completely dissolved). The mixture obtained was partitioned between hydrochloric acid (10 mL, 5% aq) and CH_2Cl_2 (3×10 mL). The combined organic layers were dried, filtered and evaporated. Without further purification, the alkylated phosphono-acetate was suspended in formaldehyde solution (1.8 mL, 18 mmol, 35% aq) and a solution of potassium carbonate (1.4 g, 10 mmol) in H_2O (2 mL) was added dropwise at rt. The reaction mixture was stirred at 80 °C for 7 h. After cooling, the reaction mixture was partitioned

between ammonium chloride (sat, 5 mL) and Et_2O (2×6 mL). The combined organic layers were washed with brine, dried and concentrated. Purification by flash chromatography (gradient 50–90% EtOAc–hexanes) afforded 443 mg (42%) of **9**. IR (film): 3105, 3095, 2978, 1713, 1631, 1601, 1519 cm^{-1}. ^1H NMR ($CDCl_3$) δ 8.13 (d, J = 7.8 Hz, 2H), 7.35 (d, J = 7.8 Hz, 2H), 6.29 (s, 1H), 5.55 (s, 1H), 4.15 (c, J = 6.8 Hz, 2H), 3.71 (s, 1H), 1.23 (t, J = 7.7 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 166.4, 147.0, 139.2, 129.9, 127.2, 123.8, 61.1, 38.3, 14.3. HRMS (M^+) for $C_{12}H_{13}O_4N$: Calcd. 235.08446, found 235.08449.

2-Triethylsilyloxymethyl-2-[(4-nitrophenyl)methyl]-oxirane (12). DIBAL-H (8.5 mL, 8.5 mmol, 1.0 M in hexane) was added dropwise to a cooled (−78 °C) solution of acrylester **9** (940 mg, 4 mmol) in THF (35 mL). After 1 h, the solution was allowed to warm up to rt. The reaction mixture was partitioned between hydrochloric acid (30 mL, 10% aq) and Et_2O (2×40 mL). The combined organic layers were washed with $NaHCO_3$ (satd), brine, dried, filtered and concentrated. The residue was filtered through silica gel (hexanes:EtOAc 1:3) to yield 760 mg (90%) of the allylic alcohol **10**: IR (film) 3366, 3084, 2919, 2848, 1648, 1596, 1614, 1437 cm^{-1}. ^1H NMR ($CDCl_3$) δ 8.13 (d, J = 8.4 Hz, 2H), 7.35 (d, J = 8.4 Hz, 2H), 5.17 (s, 1H), 4.87 (s, 1H), 4.02 (s, 2H), 3.49 (s, 2H), 1.68 (s, 1H). ^{13}C NMR ($CDCl_3$) δ 147.2, 147.0, 130.0, 123.8, 113.1, 65.4, 39.6. HRMS (M^+) for $C_{10}H_{11}NO_3$: calcd. 193.07389, found 193.07394.

Triethylamine (2.78 mL, 20 mmol), chlorotriethylsilane (20 mL, 20 mmol, 1.0 M in THF) and DMAP (15 mg, 0.12 mmol) were added to a stirred solution of alcohol **10** (4.22 g, 20 mmol) in THF (150 mL) at rt. After 3 h the solution was filtered and evaporated. The residue was purified by flash chromatography (hexanes:EtOAc 4:1) to give 6.5 g (100%) of **11**: IR (film) 3072, 2954, 2873, 1649, 1595, 1519, 1461, 1413 cm^{-1}. ^1H NMR ($CDCl_3$) δ 8.13 (d, J = 8.7 Hz, 2H), 7.34 (d, J = 8.7 Hz, 2H), 5.17 (s, 1H), 4.81 (s, 1H), 4.02 (s, 2H), 3.45 (s, 2H), 0.91 (t, J = 8.1 Hz, 9H), 0.57 (c, J = 8.1 Hz, 6H). ^{13}C NMR ($CDCl_3$) δ 147.4, 146.7, 129.8, 123.6, 112.8, 65.1, 39.4, 6.8, 4.5. HRMS (M^+) for $C_{16}H_{25}O_3NSi$: calcd. 307.16037, found 307.16046.

To a solution of silylether **11** (6.5 g, 20 mmol) in CH_2Cl_2 (200 mL) was added *m*-CPBA (9.65 g, 40 mmol, 80%). The solution was refluxed for 3 h and then stirred at rt for another 3 h. The solution was washed with $NaHCO_3$ (satd), brine, dried, filtered and concentrated. The residue was purified by flash chromatography (gradient 5–20% EtOAc–hexanes) to yield 6.5 g (95%) of **12**: IR (film) 3048, 2955, 2872, 1601, 1519 cm^{-1}. ^1H NMR ($CDCl_3$) δ 8.14 (d, J = 8.5 Hz, 2H), 7.40 (d, J = 8.5 Hz, 2H), 3.54 (s, 2H), 3.09 (s, 2H), 2.69 (d, J = 4.6 Hz, 1H), 2.53 (d, J = 4.6 Hz, 1H), 0.93 (t, J = 8.0 Hz, 9H), 0.57 (c, J = 8.0 Hz, 6H). ^{13}C NMR ($CDCl_3$) δ 147.3, 144.7, 130.9, 123.6, 65.1, 59.6, 50.4, 37.7, 6.9, 4.6.

2-Triethylsilyloxymethyl-2-[(*N*-trityl-4-aminophenyl)methyl]-oxirane (13). Pd/C (cat. 10%) was added to a solution of **12** (4.5 g, 13.25 mmol) in deoxygenated EtOAc (200 mL). The resulting solution was stirred under H_2 (1 atm) at rt. After hydrogen consumption stopped (4 h) the solution was filtered through Celite and evaporated. The residue was immediately dissolved in CH_2Cl_2 (135 mL), cooled at 0 °C and Et_3N (1.86 mL, 13.25 mmol) and tritylchloride (3.72 g, 13.25 mmol) were added. After 1 h, the solution was washed with $NaHCO_3$ (sat), brine, dried, filtered and concentrated. The residue was purified by flash chromatography (gradient 5–20% EtOAC in hexanes) to afford 6.9 g (96%) of **13**: IR (film) 3413, 3060, 3013, 2954, 2910, 2874, 1614, 1513 cm^{-1}. ^1H NMR (CD_2Cl_2) δ 7.67–7.45 (m, 15 H), 7.14 (d, J = 8.1 Hz, 2H), 6.58 (d, J = 8.0 Hz, 2H), 3.82 (d, J = 11.7 Hz, 1H), 3.23 (d, J = 11.9 Hz, 1H), 3.09 (d, J = 13.8 Hz, 1H), 2.88 (d, J = 13.9 Hz, 1H), 2.87 (d, J = 5.0 Hz, 1H), 2.73 (d, J = 4.8 Hz, 1H), 1.19 (t, J = 8.4 Hz, 9H), 0.85 (c, J = 8.4 Hz, 6H). ^{13}C NMR (CD_2Cl_2) δ 146.1, 145.5, 130.0, 129.5, 128.5, 127.1, 126.1, 116.5, 72.0, 60.6, 50.3, 37.3, 7.2, 4.9. HRMS (M^+) for $C_{35}H_{41}NSiO_2$: Calcd. 535.29066, found 535.29110.

2-Hydroxy-2-[(*N*-trityl-4-aminophenyl)methyl]-4-(2-pyridyl)-1-butanol (15). To a solution of phenyllithium (21.5 mL, 38.6 mmol, 1.8 M in cyclohexane/ether) in Et_2O (100 mL) was added dropwise (20 min) a solution of 2-picoline (3.81 mL, 38.6 mmol) in Et_2O (40 mL) The solution turned dark red. After 1 h of stirring at rt the solution was cooled to 0 °C and a solution of epoxide 13 (6.9 g, 12.85 mmol) in Et_2O (40 mL) was added over a period of 30 min (with a syringe pump). After 3 h, the reaction mixture was partitioned between ammonium chloride buffer (pH 8, sat, 150 mL) and Et_2O (2×200 mL). The combined organic layers were dried over potassium carbonate, filtered and concentrated. The residue was purified by flash chromatography (gradient 30–50% EtOAc–hexanes) to afford 5.64 g (69%) of **14**: IR (film) 3386, 3061, 3030, 2988, 1625, 1601, 1559 cm^{-1}. ^1H NMR (CD_2Cl_2): δ 8.44 (d, J = 4.2 Hz, 1H), 7.58 (dt, J = 7.8 Hz, 1H), 7.18–7.39 (m, 15H), 7.09–7.13 (m, 2H), 6.80 (d, J = 8.6 Hz, 2H), 6.30 (d, J = 8.5 Hz, 2H), 3.34 (d, J = 9.6 Hz, 1H), 3.28 (d, J = 9.6 Hz, 1H), 2.87 (m, 2H), 2.64 (d, J = 13.2 Hz, 1H), 2.56 (d, J = 13.4 Hz, 1H), 1.82 (m, 2H), 0.94 (t, J = 7.8 Hz, 9H), 0.59 (c, 7.9 Hz, 6H); ^{13}C NMR (CD_2Cl_2): δ 161.1, 149.0, 146.1, 145.4, 137.2, 130.6, 129.7, 128.4, 127.1, 126.8, 123.7, 121.6, 116.7, 72.0, 67.3, 43.0, 35.6, 32.4, 7.0, 4.6.

TBAF (2.22 mL, 2.22 mmol, 1.0 M in THF) was added at 0 °C to a solution of **14** (1.4 g, 2.22 mmol) in THF (20 mL). After 10 min, the solvent was evaporated and the residue was purified by flash chromatography (gradient 0–10% acetone–EtOAc) to yield 1.03 g (90%) of **15**: IR ($CHCl_3$) 3405, 3057, 3021, 2932, 1612, 1596 cm^{-1}. ^1H NMR (CD_2Cl_2) δ 8.43 (d, J = 4.8 Hz, 1H), 7.62 (dt, J = 7.7 Hz, 1H), 7.13–7.40 (m, 17H), 6.78 (d, J = 8.1 Hz, 2H), 6.32 (d, J = 8.7 Hz, 2H), 3.32 (d, J = 9.5 Hz, 1H), 3.28 (d, J = 9.5 Hz, 1H), 2.92 (m, 2H), 2.60 (s, 2H), 1.82 (m, 2H). ^{13}C NMR (CD_2Cl_2) δ 161.4, 149.1, 146.3, 145.5, 137.4, 130.9, 129.8, 128.5, 127.4, 126.9, 123.8, 121.8, 116.8, 72.2, 67.7, 43.1, 35.4, 32.2. HRMS (M^+) for $C_{35}H_{34}N_2O_2$: calcd. 514.26203, found 514.26215.

1,2,3,4-Tetrahydro-3-hydroxy-3-[(*N*-trityl-4-aminophenyl)-methyl]-quinolizinium methanesulphonate (16). Triethylamine (279 μL, 2.0 mmol) and methanesulfonylchloride (155 μL, 2.0 mmol) were added to a precooled (0 °C) solution of diol **15** in THF (20 mL). After 2 h, the solution was filtered, evaporated and the residue was purified by flash chromatography (5% MeOH in CH_2Cl_2). The resulting mesylate turned out to be not very stable and was therefore immediately submitted to the next step. The crude product was dissolved in acetone (100 mL) and the solution was refluxed overnight. The suspension obtained was cooled to 0 °C, the product was filtered off and dried under vacuum to yield 820 mg (70%) of **16**: IR (CH_3OH) 3402, 3055, 2934, 2516, 1632, 1613 cm^{-1}. ^1H NMR (CD_3OD) δ 8.53 (d, $J=6.3$ Hz, 1H), 8.28 (t, $J=4.4$ Hz, 1H), 7.83 (d, $J=8.4$ Hz, 1H), 7.75 (t, $J=6.8$ Hz, 1H), 7.08–7.32 (m, 15H), 6.74 (d, $J=8.8$ Hz, 2H), 6.37 (d, $J=8.3$ Hz, 2H), 4.40 (d, $J=13.5$ Hz, 1H), 4.27 (d, $J=13.5$ Hz, 1H), 3.30 (m, 2H), 2.72 (s, 2H), 2.62 (s, 3H), 1.96 (m, 1H), 1.82 (m, 1H). ^{13}C NMR (CD_3OD) δ 158.0, 147.2, 146.5, 145.8, 132.5, 131.4, 130.6, 130.0, 128.9, 128.2, 127.8, 126.3, 117.8, 72.7, 70.4, 65.6, 46.6, 39.7, 27.4. HRMS (M^+) for $C_{35}H_{33}N_2O$: Calcd. 497.25929, found 497.25952.

5-{4-[3-(1,2,3,4-Tetrahydro-3-hydroxy-quinolizinium methanesulphonate) methyl]phenyl}-amino-5-oxopentanoic acid (Hapten O2). A suspension of **16** (670 mg, 1.13 mmol) in H_2O:EtOAc (2:1, 35 mL), was vigorously stirred and refluxed until both phases clear up (2 h). The aqueous phase was washed with EtOAc (2×20 mL) and the water was azeotropically removed (coevaporation with 1:1 benzene:acetonitrile) to yield 396 mg (100%) of **17**: IR (CH_3OH) 3331, 3082, 3018, 2940, 2474, 2232, 2065, 1632, 1614 cm^{-1}. ^1H NMR (CD_3OD) δ 8.54 (d, $J=5.7$ Hz, 1H), 8.29 (t, $J=6.9$ Hz, 1H), 7.82 (d, $J=7.5$ Hz, 1H), 7.74 (t, $J=6.3$ Hz, 1H), 6.99 (d, $J=8.4$ Hz, 2H), 6.63 (d, $J=8.4$ Hz, 2H), 4.47 (d, $J=14.1$ Hz, 1H), 4.33 (d, $J=13.2$ Hz, 1H), 3.31 (m, 2H), 2.83 (s, 2H), 2.62 (s, 3H), 2.01 (m, 1H), 1.87 (m, 1H); ^{13}C NMR (CD_3OD) δ 157.8, 147.9, 146.3, 145.7, 132.4, 129.4, 126.4, 125.8, 116.6, 70.3, 65.4, 65.3, 46.4, 39.6, 27.3. HRMS (M^+) for $C_{16}H_{19}N_2O$: calcd. 255.14974, found 255.14975.

To a solution of the resulting aniline quinolizinium salt **17** (396 mg, 1.13 mmol) in acetonitrile (15 mL) was added glutaric anhydride (193 mg, 1.64 mmol) at rt. After 36 h, the solvent was evaporated and the residue redissolved in water. The aqueous solution was washed with EtOAc and then azeotropically evaporated (coevaporation with 1:1 benzene:acetonitrile) to yield 460 mg (88%) of hapten **O2**: IR (CH_3OH) 3412, 3107, 3048, 2931, 2647, 1719, 1666, 1628 cm^{-1}. ^1H NMR (CD_3OD) δ 8.54 (d, $J=6.3$ Hz, 1H), 8.29 (t, $J=7.7$ Hz, 1H), 7.83 (d, $J=8.1$ Hz, 1H), 7.73 (t, $J=6.5$ Hz, 1H), 7.43 (d, $J=8.4$ Hz, 2H), 7.18 (d, $J=8.4$ Hz, 2H), 4.48 (d, $J=14.1$ Hz, 1H), 4.29 (d, $J=14.1$ Hz, 1H), 3.31 (m, 2H), 2.90 (s, 2H), 2.58 (s, 3H), 2.28 (m, 4H), 2.02 (m, 1H), 1.86 (m, 2H), 1.78 (m, 1H); ^{13}C NMR (D_2O) δ 180.7, 177.5, 158.5, 147.4, 147.2, 138.5, 134.8, 134.0, 131.2, 127.8, 124.7, 72.1, 67.0, 66.0, 47.2, 41.1, 38.1, 35.7, 31.5, 28.3. HRMS (M^+) for $C_{21}H_{25}N_2O_4$: calcd. 369.18143, found 369.18092.

1,2,3,4-Tetrahydro-2-[(4-nitrophenyl)methyl]-isoquinoline (19). To a stirred suspension of NaH (0.5 g, 16.5 mmol, 80%) in THF (20 mL) was added dropwise 1,2,3,4 tetrahydro-isoquinoline **18** (1.9 mL, 15 mmol). The resulting mixture was stirred at rt for 3 h. A solution of 4-nitrobenzyl bromide (3.24 g, 15 mmol) in THF (20 mL) was added dropwise and the reaction mixture was stirred overnight. The reaction mixture was filtered, concentrated and purified by flash chromatography to afford 3.76 g (94%) of **19** as a yellow solid. ^1H NMR ($CDCl_3$) δ 8.83, (d, $J=8.7$ Hz, 2H), 7.58 (d, $J=8.7$ Hz, 2H), 7.20–7.05 (m, 3H), 7.00–6.95 (m, 1H), 3.77 (s, 2H), 3.65 (s, 2H), 2.92 (t, $J=6.0$ Hz, 2H), 2.67 (t, $J=6.0$ Hz, 2H); ^{13}C NMR ($CDCl_3$) δd 146.5, 134.3, 134.1, 129.4, 128.7, 126.5, 126.3, 125.7, 123.6, 77.4, 77.0, 76.6, 61.8, 56.1, 50.8, 29.1.

1,2,3,4-Tetrahydro-2-[(4-aminophenyl)methyl]isoquinoline (20). A mixture of **19** (1.67 g, 6.23 mmol), Pd/C (cat. 10%) and hydrazine hydrate (1.0 g, 20 mmol) in isopropyl alcohol (20 mL) was refluxed for 24 h. After cooling, the solution was decanted, concentrated and purified by flash chromatography. Recrystallization from acetonitrile afforded 1.27 g (86%) of **20** as a white solid. IR (film) 2902, 2799, 2352, 1600, 1496, 1462 cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.27 (s, 1H), 8.19 (s, 1H), 7.13 (d, $J=8.4$ Hz, 2H), 7.10–7.02 (m, 3H), 3.52 (s, 2H), 3.32 (s, 2H), 2.78 (t, $J=6.1$ Hz, 2H), 2.64 (t, $J=6.1$ Hz, 2H); ^{13}C NMR (DMSO-d_6) δ 151.1, 135.0, 134.2, 129.7, 129.1, 128.9, 128.4, 126.3, 125.9, 125.8, 125.4, 112.6, 61.8, 55.4, 50.1, 28.8.

5-{4-[2-(1,2,3,4-Tetrahydroisoquinolyl)methyl]phenyl}amino-5-oxo-pentanoic acid (21). A solution of **20** (1.0 g, 4.2 mmol) and glutaric anhydride (0.57 g, 5.0 mmol) in EtOAc (40 mL) was stirred for 1 h (open to air atmosphere). The reaction mixture was concentrated and purified by flash chromatography (gradient 15–50% MeOH–CH_2Cl_2) to yield 1.45 g (98%) of **21** as a white solid which was recrystallized from EtOAc/hexanes. ^1H NMR (DMSO-d_6) δ 9.88 (s, 1H), 7.41 (d, $J=8.4$ Hz), 7.25 (d, $J=8.4$ Hz), 7.12–7.02 (m, 3 H), 7.02–6.95 (m, 1 H), 3.55 (s, 2H), 3.16 (s, 2H), 2.78 (t, $J=6.1$ Hz, 2H), 2.64 (t, $J=6.1$ Hz, 2H), 2.33 (t, $J=7.8$ Hz, 2H), 2.26 (t, $J=7.2$ Hz, 2H), 1.79 (q, $J=7.4$ Hz, 2H). ^{13}C NMR (DMSO-d_6) δ 173.6, 170.1, 137.6, 134.2, 133.6, 132.2, 128.5, 127.8, 125.8, 125.4, 124.9, 118.4, 60.9, 54.8, 49.6, 34.9, 32.5, 28.1, 20.0. HRMS (M^+) for $C_{21}H_{24}N_2O_3$: calcd 354.4332, found 354.4335.

5-{4-[2-(1,2,3,4-Tetrahydroisoquinolyl-*N*-oxide)methyl]-phenyl}amino-5-oxo-pentanoic acid (hapten O3). To a stirred solution of **21** (0.446 g, 1.27 mmol) in THF (10 mL) was added *m*-CPBA (386 mg, 1.9 mmol, 85%) over 2 min. After 10 min, the reaction was complete and the precipitated product was filtered and the residue washed with THF and CH_2Cl_2, and dried under vacuum to give 400 mg (97%) of hapten **O2**: ^1H NMR (DMSO-d_6) δ 10.13 (s, 1H), 7.63 (d, $J=8.4$ Hz, 2H), 7.49 (d, $J=8.4$ Hz, 2H), 7.23–7.10 (m, 3H), 6.98 (d, $J=8.2$ Hz, 2H), 4.64 (s, 3H), 4.17 (d, $J=15.1$ Hz, 2H), 3.70–3.54 (m, 3H), 3.34–3.18 (m, 3H), 2.32 (t, $J=7.4$ Hz, 2H), 2.23 (t, $J=7.4$ Hz, 2H), 1.83–1.69 (m,

2H). ^{13}C NMR (DMSO-d_6) δ 175.5, 171.3, 140.4, 133.4, 131.5, 129.1, 128.1, 127.1, 126.8, 126.2, 123.8, 118.5, 71.0, 67.0, 64.0, 35.8, 34.5, 25.1, 21.0; HRMS (M^+) for $C_{21}H_{24}N_2O_4$: calcd. 370.4326, found 368.4323.

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