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Multiple Reactive Immunization Towards the Hydrolysis of Organophosphorus Nerve Agents: Hapten Design and Synthesis

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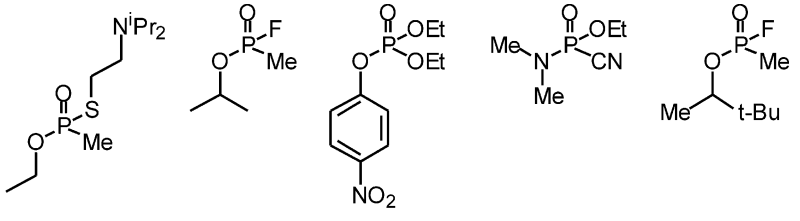
Abstract—We designed and synthesized a series of haptens to elicit catalytic antibodies with phosphatase activity against nerve agents. The design is based on the novel concept of multiple reactive immunization which aims to afford two or more catalytic residues within the antibody's binding cleft. The haptens showed the desired reactivity in vitro and were submitted for immunization.

Introduction

Protection against organophosphorus nerve agents is a matter of international concern. 'Nerve agent', or 'nerve gas', represents a generic name for a class of highly toxic compounds with marked inhibitory activity on the enzyme acetylcholinesterase. Some organophosphorus compounds (e.g., sarin, paraoxon, tabun, soman, and others) displaying extremely toxic activity are widely used as insecticide toxins and are also present in warfare arsenals as effective chemical weapons. The dangers and lethality of the chemical arsenal render a sense of urgency to the search for potentially effective detoxifying agents.

Many efforts have been invested towards the safe destruction of these nerve agents.[1] Nevertheless, efficient environmentally compatible solutions are yet to be achieved. The efficient neutralization of these toxin molecules in vivo before they hit their target is also in demand. Even though antidotal in vivo treatment with atropine derivatives and oximes is well known their beneficial effects are limited.[2] Phosphotriesterase enzymes and some of their mutants reportedly have hydrolyzed a variety of organophosphate insecticides and acetylcholinesterase inhibitors.[3] Catalytic antibodies[4] with phosphatase-like activities elicited from transition state analogues have also been proposed as detoxifying and neutralization tools.[5] In this paper we describe a novel approach to the development of catalytic antibodies with a wide phosphatase activity.

Antibody catalyzed reactions take place in an aqueous environment, close to ambient temperature and at neutral pH. These efficient catalysts are environmentally friendly in their nature. Due to its protein character, this class of biocatalysts is potentially biodegradable. Evidently, biodegradation of the detoxifying agent after decontamination, neutralization or destruction of the toxic compound represents an optimal post-treatment opportunity.



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Monoclonal antibodies can be fully humanized[6] and in principle can be used for therapeutic purposes.[7] Hence, antibodies catalyzing the hydrolysis of toxic organophosphorus compounds will be of pharmacological importance in the prevention of neurotoxin poisoning.

In contrast to ordinary immunization, where the elicited antibody arranges amino acids within its binding site generating the required shape and charge complementarity to the challenging non-reactive antigen, reactive immunization is carried out with reactive compounds in order to promote a chemical reaction during the binding of the antigen to the antibody. Thus, following the initial binding event, the hapten might encounter an occasional reactive group in the binding site yielding a covalent bond between the antigen and the antibody. The reactive immunization process is dependent on the reactivity of the hapten as well as that of the antibody. The binding energy achieved in this step is greater than otherwise possible and the somatic mutations will presumably stop at about this point. Consequently, the resulting antibodies may present relatively promiscuous binding pockets that may accept broad ranges of substrates. Further mutations could lead to antibodies that are more reactive than those generated by the primary response and result in faster reactions and tighter binding.

The reactive immunization technique represents a major step towards the generation of antibodies bearing desired catalytic residues approaching enzymatic performance.[8] The power of this technique has been nicely demonstrated by eliciting antibodies that catalyze the aldol condensation reaction using the same mechanism as the class I aldolase enzymes.[8c] Aldolase catalytic antibodies approximate the rate acceleration of the natural enzymes used in glycolysis.[8c] Antibodies elicited via reactive immunization are very broad on scope. More than 100 aldehyde–aldehyde, aldehyde–ketone, and ketone–ketone aldol addition reactions have been catalyzed by aldolase antibodies. Some of these reactions yielded the key pieces for the total synthesis of natural products.[9] While aldolase antibodies are very efficient, phosphatase antibodies only achieved modest activities. This work focuses on meeting the challenge of generating efficient phosphatase antibodies.

The haptens described in this work are designed to elicit antibodies via 'multiple' reactive immunization, leading to two or more catalytic residues strategically located in the binding site. In principle, haptens with different reactivities will afford different catalytic groups (e.g., Lys, Ser, Thr, Tyr, Cys, His) in the elicited biocatalysts. In general, these haptens present only one perceptible reactive center that upon reaction with the antibody unleashes a second latent reactive center which is then ready to capture a second nucleophilic residue within the antibody's binding site (Scheme 1).

These haptens consist of two detachable parts: one carrying a reactive center (triangle reactive center attached to A in Scheme 1) and another one bearing a second tacit reactive center as well as the carrier protein connected through an appropriate linker (square reactive center attached to B in Scheme 1). The relative positioning of the reactive centers in the hapten will dictate the location of the catalytic groups in the raised antibodies. Catalytic antibodies elicited by this method will be easily identified by a rather simple Enzyme-Linked Immunosorbent Assay (ELISA) test. Selected antibodies binding to the challenging hapten will hold the promise of being efficient catalysts for the first reaction and in addition will present a second nucleophilic group within the active site. Lack of the second reaction and diffusion away from the antibody would result in the neutralization of the second piece by a solvent molecule and no evident binding of the hapten to the antibody. The antibodies generated via double (or multiple) reactive immunization are expected to be effective catalysts for the hydrolysis of a wide range of organophosphate (phosphodi- and triesters) and organophosphonate compounds in addition to the above mentioned organophosphorus toxic agents and their analogues.

The mechanism of the antibody catalyzed reactions cannot be anticipated with great reliability. On the one hand, it is possible that the organophosphorus substrates will phosphorylate the binding site yielding covalent adducts. On the other hand, the particular electrostatic environment and amino acid side chains within the antibody's binding site may activate molecules of water and therefore hydrolyze the substrate via either an associative or a dissociative mechanism[10] resulting in a catalytic process with multiple turnovers.[11]

Results and Discussion

Design and synthesis of a phosphonate hapten for double reactive immunization

Following the premises described above we designed the first hapten in this series. Hapten **7** presents a soft-shell labile phosphonate center that after hydrolytic nucleophilic attack triggers the latent quinone methide as the second reactive species. Hapten **7** was prepared as depicted in Scheme 2. Thus, the commercially available 4-hydroxymandelic acid (**1**) reacted with 5-aminohexanoic acid, which was previously protected as its corresponding benzyl ester. The resulting phenol (**2**) reacted with methyl ethyl phosphonic chloride, generated in situ from ethyl phosphonic dichloride and one equivalent of methanol. Subsequent substitution of the secondary OH group with fluoride using the DAST reaction afforded the precursor (**4**) for hapten **7**. The carboxy group in **4** was deprotected by hydrogenation and then activated as the corresponding *N*-succinimide derivative prior to coupling to the carrier protein and immunization.

When **4** was incubated with an excess of *N*-butylamine in a solution of 95% dioxane in water, emulating the conditions within the hydrophobic binding site of a typical antibody,[12] it afforded the expected derivative (**6**) which was isolated by HPLC and characterized by ESI-MS (calcd for MNa^+: 346, found: 346). This

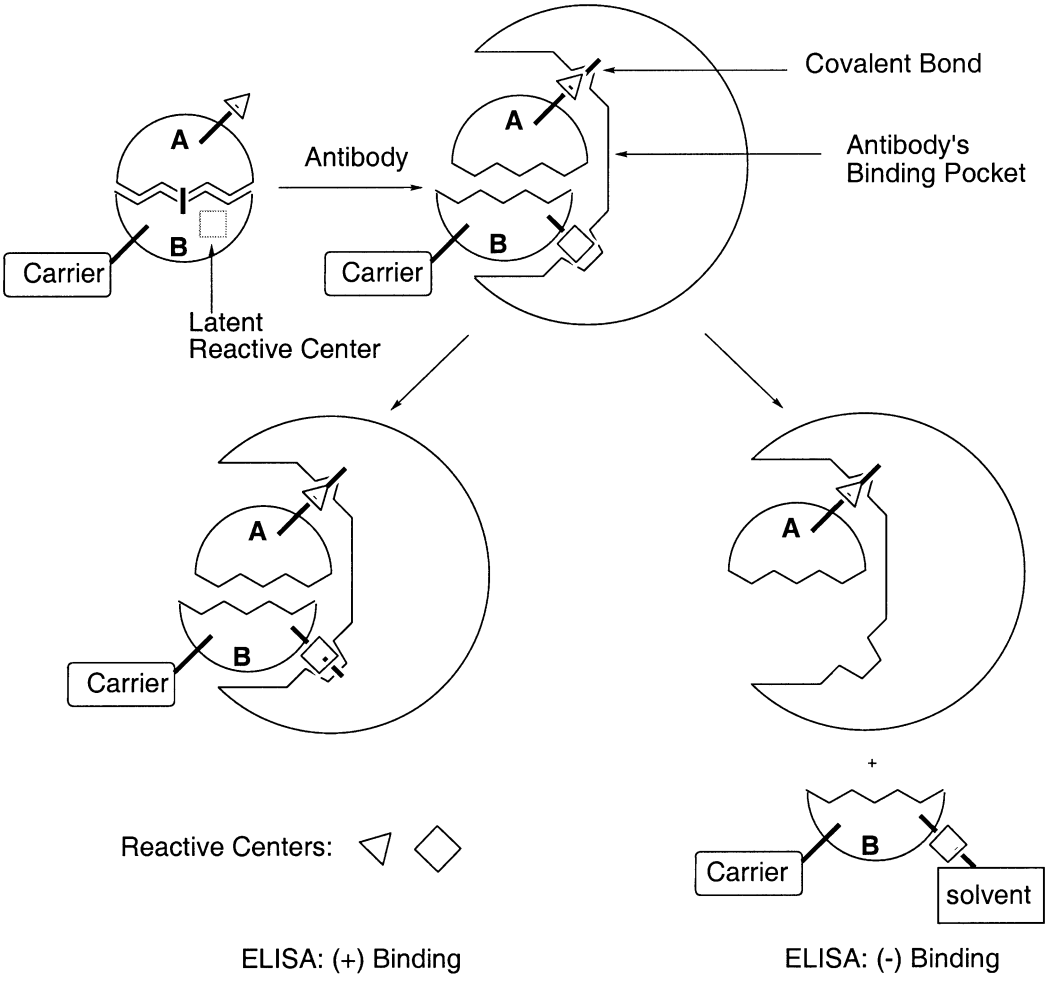
compound is the result of two nucleophilic attacks on **4**, the first (nucleophile = water or amine) on the phosphorus atom and the second (nucleophile = amine) on the quinone methide generated in situ after the first nucleophilic attack, in addition to the hydrolysis of the benzyl ester moiety under the reaction conditions. We expect hapten **7** to react similarly with nucleophilic residues in the binding sites of the elicited antibodies. The first nucleophilic group (i.e., a nucleophilic group from an amino acid side chain or an activated water molecule) in the antibody's binding site will be phosphorylated, and the second one will react with the quinone methide after elimination of a fluoride ion (Scheme 3). Since the linker-carrier protein construct is attached to the quinone methide moiety, the success of the double reactive immunization will be readily monitored by an ELISA test.

A suicide inhibitor, 4-(fluoromethyl)phenyl phosphate, acting through a similar mechanism as described above for **7**, was successfully used to inactivate phosphotyrosine phosphatase.[13] The relative stability of this inhibitor under physiological conditions coupled with its reactivity towards the target enzyme are features that gives further support to the feasibility of our hapten design.

Design and preparation of a phosphonate hapten for multiple reactive immunization

Based on a similar general strategy to that used in the design of hapten **7**, the system could be pushed one step further by the generation of antibodies with three active site nucleophilic residues (Scheme 4).

For this purpose, a second hapten was designed including a phosphonate center with two differentially labile phenolates. One phenol moiety with an electron-withdrawing group (EWG) and a second phenol carrying a latent quinone methide species. In this case, the first hydrolysis will result in the release of the phenol including the EWG (the more labile substituent attached to the phosphorus atom) and the monophosphorylation of the antibody. This putative antibody-phophonate will then undergo a second nucleophilic attack triggering the quinone methide group. A third nucleophile will then react with the last fragment bearing the carrier protein and be readily identified by a simple ELISA test. Alternatively, if the first hydrolysis is caused by a water molecule, the resulting phosphonate could either diffuse away from the binding site or react with a nucleophilic residue.



Scheme 1. Schematic representation of the multiple reactive immunization concept.

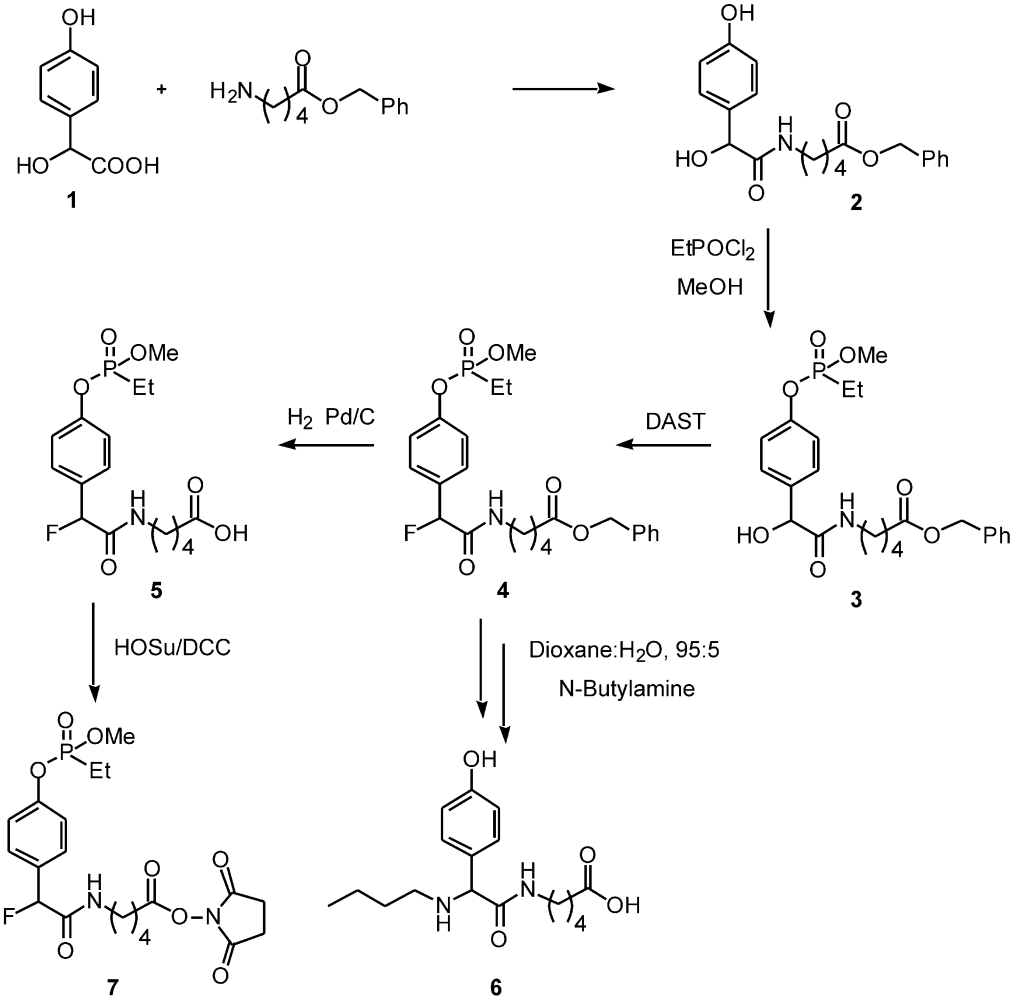
The key design feature in this hapten is the sequential hydrolysis of the phenol substituents on the central phosphorus atom. The described course of events developed during the immunization process is likely to bestow antibodies including two residues participating in a phosphonate moiety as well as a third nucleophilic residue that would react with the quinone methide formed in situ.

To the best of our knowledge the experimental K_{hydrol} values for compounds structurally related to our hapten design are not fully available. Assuming that the O–P bond strength of given phenol phosphates correlates with the O–H acidity of the corresponding phenols we turned to the computational estimation of the pK_a values of the later species. We reasoned that the most acidic phenol would constitute a more easily hydrolyzable group in the phosphonate hapten, accounting for the required cleavage sequence.

We used two methods for our pK_a predictions. The first one is the Schrödinger's Jaguar pK_a module,[14,15] which performs with a mean absolute error of 0.25 compared to experimental results.[14] For further assessing the unknown pK_a values, we also performed calculations using another relatively straightforward method.[16,17] In this method, the geometry optimization calculations were carried out using the Amsterdam Density Functional (ADF, Version 1999) package.[18] For solvation energy calculations, we used the MEAD (Macroscopic Electrostatics with Atomic Detail) program suite developed by Bashford.[19]

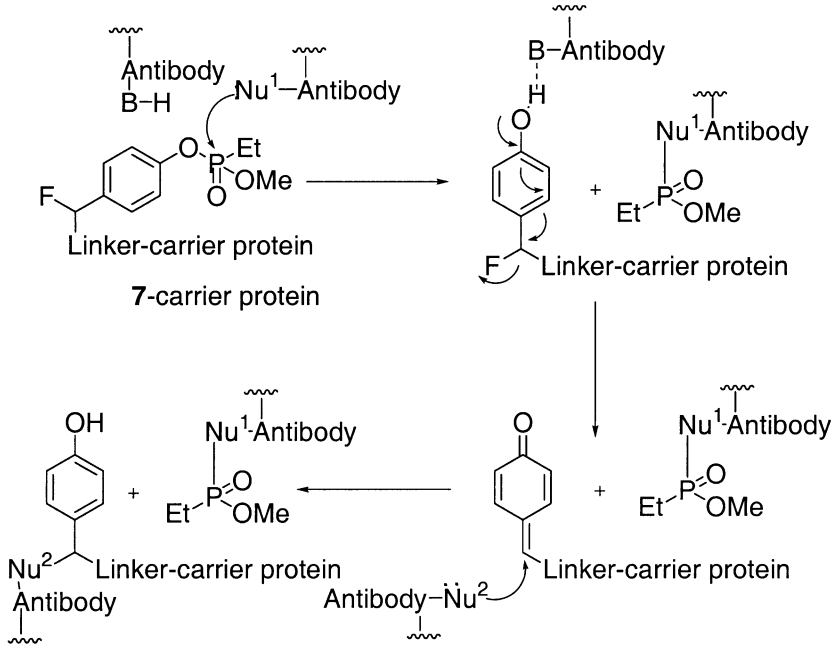
ADF/MEAD is a simpler and faster alternative to performing full ADF/SCRF calculations for pK_a's.[16,17,22,26,27] We compared the two methods for **8**. ADF/SCRF gives a pK_a = 10.3[16a,17] using the constants given in eq (2) (see computational methods in the Experimental). This value is one pK_a unit higher than that obtained from ADF/MEAD calculation (pK_a = 9.3). These two results bracket the experimental value of pK_a = 10.0.

We calculated the pK_a's of a series of 29 phenols (Table 1 and Fig. 1) including 17 with reported acidity constants in water at 25 °C.[20] The predicted pK_a values correlate very well with the reported experimental results (Table 1) further endorsing the estimated unknown pK_a values. Table 1 shows that the ADF/MEAD method predicts the same pK_a serial order for the different phenols as the experimental data and the

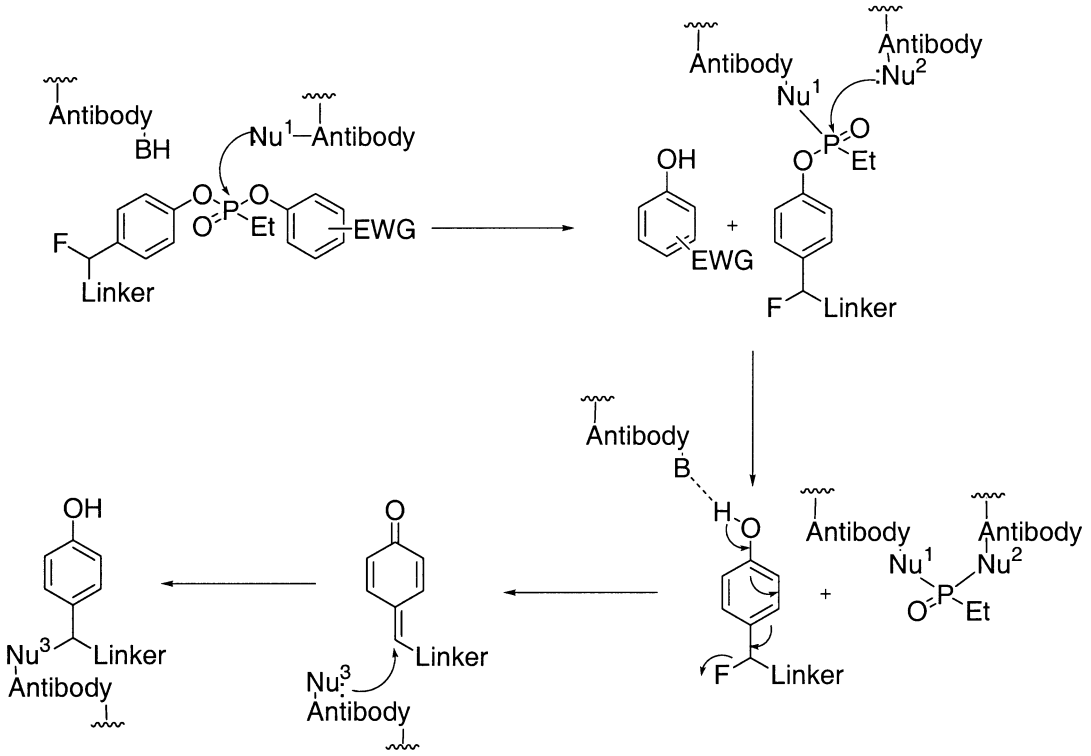


Scheme 2. Synthesis of hapten **7**.

Jaguar results with a few exceptions. The only deviations were found for the phenols with $-NO_2$ (**9**, **10** and **11**) and $-CF_3$ groups (**20** and **21**). It is highly significant for our purposes that both theoretical methods predict the largest pK_a value for **36**, and the smallest pK_a values for phenols **10** and **11**.[21] The relative predicted ordering of the pK_a's of the above mentioned phenols as well as the predicted pK_a shift (ΔpK_a = 1.5 for Jaguar and 1.8 for ADF/MEAD) are in good agreement between the two methods. The ideal combination of phenol substituents to the phosphonate core of the designed hapten would include the most and the least acidic phenols to guarantee the programmed sequential cleavage. Disregarding stability considerations, a hapten including the combination of phenols **36** and **10** or **11** would be the most suitable for our purposes.



Scheme 3. Double reactive immunization with hapten **7**.



Scheme 4. Multiple reactive immunization leading to three different nucleophile catalytic residues in the antibody binding site.

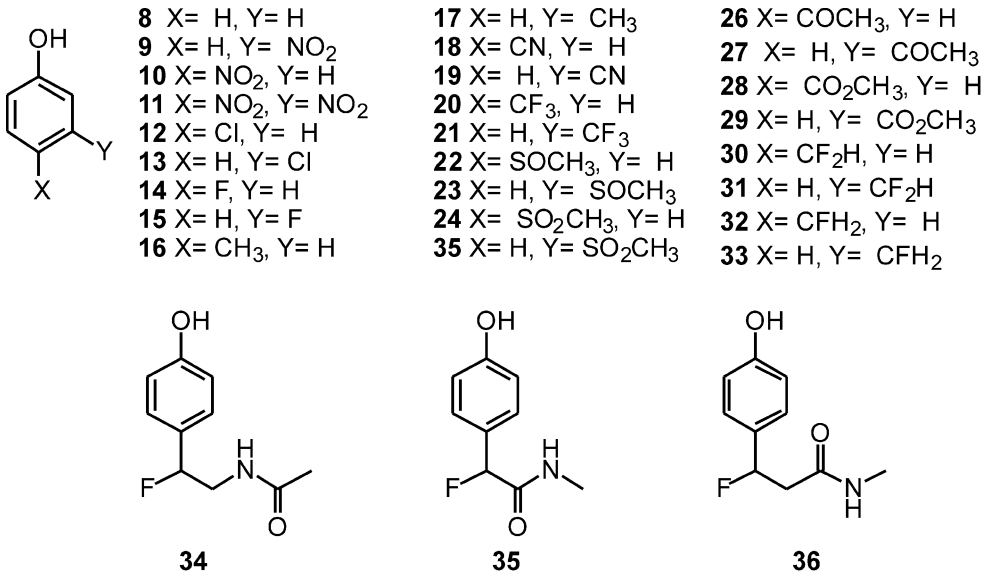


Figure 1. Structures of phenols used for pK_a calculations.

Table 1. Experimental and calculated pK_a values for the selected phenols

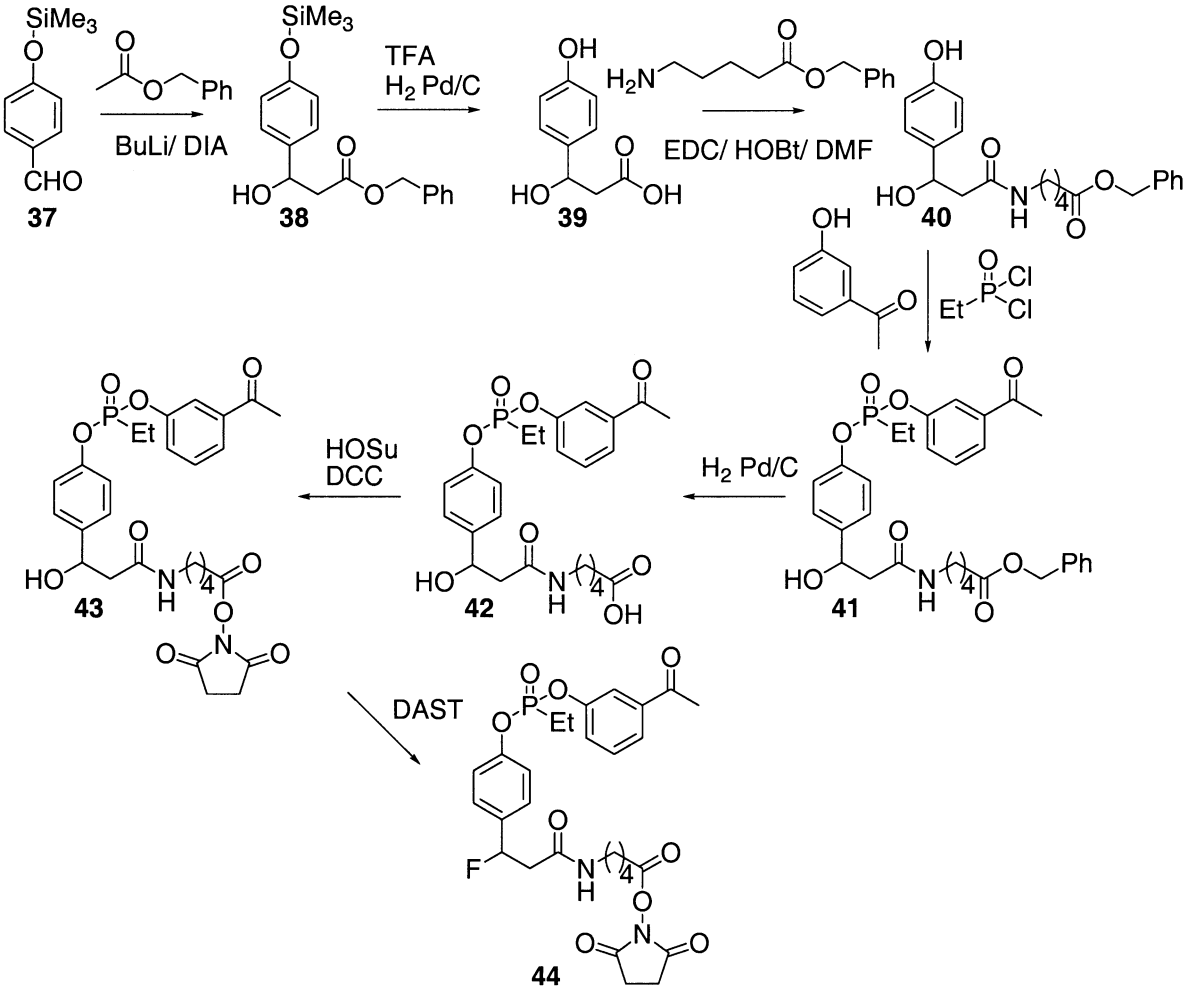
Phenol	Exp. data	Jaguar	ADF/MEAD	Phenol	Exp. data	Jaguar	ADF/MEAD
8	9.98	9.8	9.3	**23** ($mSOCH_3$)	8.75	9.4	8.3
9 (mNO_2)	8.38	8.6	7.3	**24** (pSO_2CH_3)	7.83	8.2	6.2
10 (pNO_2)	7.16	7.1	3.5	**25** (mSO_2CH_3)	9.33	9.3	8.3
11 (m,pNO_2)	5.22	5.7	4.1	**26** ($pCOCH_3$)	8.05	8.2	8.5
12 (pCl)	9.42	9.6	8.2	**27** ($mCOCH_3$)	9.19	9.3	9.7
13 (mCl)	9.08	9.5	7.5	**28** (pCO_2CH_3)		8.8	7.5
14 (pF)	9.89	9.8	9.4	**29** (mCO_2CH_3)		9.6	11.3
15 (mF)	9.29	9.1	7.7	**30** (pCF_2H)		9.3	8.6
16 (pCH_3)	10.25	10.3	11.2	**31** (mCF_2H)		9.5	9.5
17 (mCH_3)	10.08	9.8	10.3	**32** ($pCFH_2$)		9.5	11.0
18 (pCN)	7.80	8.0	5.4	**33** ($mCFH_2$)		9.5	11.1
19 (mCN)		8.6	7.2	**34**		10.2	9.3
20 (pCF_3)	8.68	9.3	7.8	**35**		10.4	11.3
21 (mCF_3)		9.1	9.3	**36**		10.8	11.5
22 ($pSOCH_3$)	8.28	9.0	6.5				

The relative stability of the hapten under physiological conditions combined with its sequential reactivity is pivotal in our approach. Therefore, the delicate balance between reactivity and stability of the antigen directed our synthetic efforts. After several synthetic attempts experimenting with different phenols (e.g., **24**, **27**, **28**, and **29**), we successfully completed the preparation of the designed hapten using the combination of phenols **36** and **27** (Scheme 5). All other attempts resulted in unstable compounds that would not be appropriate for immunization.

Thus, 3-hydroxy-3-(4-hydroxy-phenyl)-propionic acid (**39**) was obtained after deprotection of the aldol product between 4-(trimethyl-silanyloxy)-benzaldehyde (**37**) and acetic acid benzyl ester. The key phosphonate intermediate (**41**) was prepared after the installation of a five-carbon chain linker. Selective cleavage of the benzyl ester followed by succinimide activation of the resulting acid and DAST reaction at $-78\,^{\circ}C$ (carefully followed by TLC) afforded **44** in reasonable yield. The latter reaction sequence efficiently reduced the appearance of elimination side products. Hapten **44** is relatively stable with an estimated half-life longer than 48 h.

Cleavage of the 3-Hydroxyacetophenone moiety of **44** under mild aqueous acidic conditions was monitored by HPLC. This reaction represents the first step in the reactivity of this hapten as hitherto described in Scheme 4 and is in agreement with the predicted relative reactivity of **27** and **36**. This result which can then be followed by the hydrolysis of the resulting phosphonate confirms the soundness of our hapten design. The chemical properties of **44** indicate that it is suitable for the planned immunization.

In the event of a successful immunization with hapten **44**, the elicited antibodies will include two residues participating in a phosphonate moiety. This potential 'chelating site', determined by the two nucleophilic residues, could potentially result in catalytic metalloantibodies. The chemistry of these intriguing bioorganometallic complexes will be explored.



Scheme 5. Synthesis of hapten **44**.

Conclusions

We accomplished the synthesis of two haptens for multiple reactive immunization. The haptens were designed to react, and therefore elicit, with more than one nucleophile in the antibody binding cleft. Haptens **7** and **44** were recently conjugated with ovalbumin and bovine serum albumin (BSA) and submitted for immunization. The activities of the elicited catalytic antibodies will be reported in due course.

In our program, we seek to induce antibodies with multiple functionalities arranged in optimal positions to facilitate catalysis. The generation of two or more catalytic residues with the ability to communicate through hydrogen bonding or transfer is of great importance in enzyme mimetics design since such a feature is key to many enzymatic processes. Thus, the synthesis of a second set of haptens with a latent *o*-quinone methide is under way.

Experimental

General remarks

Reagents were purchased from Aldrich. Thin layer chromatography (TLC): silica gel plates Merk 60 F_{254}, compounds were visualized by irradiation with UV light and/or by treatment with a solution of phosphomolybdic acid followed by heating or by immersing in an iodine bath. Flash chromatography: silica gel Merk 60 (particle size 230–400 mesh) eluent given in parentheses. NMR: Bruker AMX 300, Bruker AMX 400, The chemical shifts (δ) are given in ppm, and the coupling constants *J* are given in Hz. The NMR spectra were recorded in $CDCl_3$ unless stated otherwise.

Computational methods

We used two methods for our pK_a predictions. The first one is the Schrödinger's Jaguar pK_a module.[14,15] This module utilizes ab initio quantum chemical methods to predict pK_a's in aqueous media. It employs a combination of correlated ab initio quantum chemistry, self-consistent reaction field (SCRF), continuum treatment of solvation,[22] and systematic corrections to account for approximations in both the ab initio and continuum solvation methods. In this method, the geometry optimization calculations are performed at the B3LYP/6-31G** level of theory. Then a larger basis set cc-pVTZ++ is used to get the single point energies. Zero point energies are estimated and the solvation free energies are obtained through the SCRF procedure.[22]

Correction factors are derived from a simple linear form.[14] After all the systematic corrections are added, the calculation yields good agreement with experimental results as described in the Jaguar manual. In the particular calculations for the test set of phenols, Jaguar pK_a's module performs with a mean absolute error of 0.25 compared to experimental results.[14]

For further assessing the unknown pK_a values, we also performed calculations using another relatively straightforward method.[16,17] In this method, the geometry optimization calculations were carried out using the Amsterdam Density Functional (ADF, Version 1999) package.[18] The parametrization of Vosko, Wilk and Nusair[23] was used for the local density approximation term, and the Becke[24] and Perdew[25] nonlocal corrections were used for the nonlocal exchange and correlation terms. The molecular orbitals were expanded in an uncontracted triple-ζ Slater-type orbital basis set, along with a single set of polarization functions, which constitutes a basis set IV in the ADF code. The inner core shells were treated by the frozen core approximation.

After the geometry optimization, we used Chargefit, a modified version of the CHELPG code[26,27] to fit the point charges from the molecular electrostatic potentials (ESP) calculated by ADF. The total net charge of the molecule and the three Cartesian dipole moment components from density functional calculations were adopted as constraint conditions for the fit. The fitted points lay on a cubic grid between the van der Waals radius and the outer atomic radius with a grid spacing of 0.2 Å. The outer atomic radius for all atoms used was 5.0 Å, and the van der Waals radii for C, O, N, H, F, Cl, and S were 1.67, 1.4, 1.55, 1.2, 1.7, 1.9 and 1.8 Å, respectively. In order to minimize the uncertainties in the fitting procedure, the singular value decomposition (SVD) method[27] was introduced into the code to obtain a model with stable atomic charges and an accurate molecular dipole moment. For solvation energy calculations, we used the MEAD (Macroscopic Electrostatics with Atomic Detail) program suite developed by Bashford.[19] In this approach, the solute–solvent interaction is treated within the framework of classical electrostatics. The solute is represented by a set of atomic charges and Born radii, and solvent as a continuous dielectric medium (with $\varepsilon = 80.0$). The dielectric boundary between the interior and the exterior is defined by the surface of contact of a 1.4 Å sphere rolling over the superposition of spheres defined by the Born radii of the atoms. The free energy difference for charging the solute in vacuum and in solution is calculated by solving the macroscopic Poisson equation using a numerical finite-difference method. We used the same radii for the various atoms as in the charge-fit calculations, except for the C, N and O atoms with 2.0 Å in the $-NO_2$ and –CN groups. These radii were made larger due to the multiple bond character of the $-NO_2$ and –CN groups (in agreement with the Jaguar radii[22]).

Finally, we calculated the pK_a associated with the process [eqs (1) and (2)]:

$$AH \rightarrow A^- + H^+, \tag{1}$$

$$\begin{aligned} 1.37pK_a &= \{E(A^-) + E(H^+) - E(AH) + E_{corr}\} \\ &\quad + \{G_{sol}(A^-) - G_{sol}(AH)\} - 268.26 \\ &= PA + \Delta G(_{deprot}) - 268.26 \end{aligned} \tag{2}$$

In eq (2) $E(A^-)$, $E(H^+)$ and E(AH) represent the gas-phase energies of the deprotonated phenol, proton and neutral phenol, respectively. E_{corr} is a correction term to the proton affinity PA, including zero point energy (ΔZPE) and 5/2RT work. We only calculated the $\Delta ZPE = -8.8$ kcal/mol for phenol (**8**). For simplicity, we then used $\Delta ZPE = -8.8$ kcal/mol through all the pK_a calculations. $G_{sol}(A^-)$ and $G_{sol}(AH)$ are the solvation free energies for the deprotonated phenol and phenol, respectively -268.26 comes from the sum of the solvation free energy of a proton (-260.5 kcal/mol)[19,28–30] (using the estimated value of Noyes),[29] and the translation entropy contribution to the gas-phase free energy of a proton ($-T\Delta S_{gas}(H^+) = -7.76$ kcal/mol at 298K and 1 atm pressure).[28]

5-Aminovaleric benzyl ester. 5-Aminovaleric acid hydrochloride (7.68 g, 50 mmol) and 4-toluenesulfonic acid monohydrate (10.46 g, 55 mmol) were added to freshly distilled benzyl alcohol (25 mL) in a 250 mL round bottle flask equipped with a Dean–Stark trap. The mixture was refuxed until all the water was removed from the reaction mixture. The mixture was then cooled to room temperature. Ethyl ether (500 mL) was added to the mixture and cooled down to 0 °C for 2 h. Crystalline 5-aminovaleric benzyl ester salt was collected by filtration. After recrystallization from ether/ethyl acetate. The white crystalline salt (11.2 g) was collected in 91% yield. Spectroscopic data was in agreement with that reported in the literature.[31]

5-[2-Hydroxy-2-(4-hydroxy-phenyl)-acetylamino]-pentanoic acid benzyl ester (2). DL-4-hydroxymandelic acid (2 g, 11.9 mmol), 1-hydroxybenzotriazole monohydrate (1.82 g, 11.9 mmol), Diisopropylethylamine (3.85 g, 29.8 mmol) and 5-aminovaleric benzyl ester (5.68 g, 13.1 mmol) were dissolved in DMF (50 mL). (3-dimethyaminopropyl)-3-ethylcarbodiimide hydrochloride anhydrous (EDC, 2.281 g, 11.9 mmol) was added and the solution was stirred at ambient temperature for 24 h. The DMF was then removed under reduced pressure and the residue dissolved in ethyl acetate (50 mL). After washing with water, citric acid and brine, the organic phase was dried over anhydrous Na_2SO_4. Flash column chromatography (ethyl ether:ethyl acetate) afforded the purified product (3.1 g, 72% yield). ESI-MS: calcd for $C_{20}H_{23}NO_5$ MH^+: 358.2, found: 358. ^{1}H NMR (400 MHz) δ 1.53 (m, 2H), 1.62 (m, 2H), 2.35 (t, 2H), 3.23 (m, 2H), 4.88 (s, 1H), 5.09 (s, 2H) 6.58 (d, 2H, $J = 8.5$ Hz), 7.04 (d, 2H, $J = 8.5$ Hz), 7.34 (m, 5H) ppm. ^{13}C NMR (100.6 MHz) δ 14.20, 21.70, 28.80, 33.60, 38.90, 60.70, 66.40, 73.70, 115.7, 128.3, 128.6, 130.6, 135.8, 156.4, 173.8 ppm.

5-{2-[4-(Ethyl-methoxy-phosphinoyloxy)-phenyl]-2-hydroxy-acetylamino}-pentanoic acid benzyl ester (3). Diisopropylethylamine (520 mg, 4 mmol) was added to a solution of ethyl phosphonate dichloride (293.8 mg, 2 mmol) in CH_2Cl_2 (10 mL). Methanol (64 mg, 2 mmol) was added and the solution was stirred at ambient temperature for 1 h. DL-4-Hydroxymandelic-5-amide valeric benzyl ester (714 mg, 2 mmol) was added followed by diisopropylethylamine (390 mg, 1.50 mmol). After stirring for 3 h at ambient temperature, the reaction mixture was washed with brine and the solvents removed under reduced pressure. The residue was purified by flash column chromatography using a stepwise gradient strarting with ethyl ether/ethyl acetate and ending with ethyl acetate/acetoniltrile. Purification afforded 509 mg of product (55% yield). ESI-MS: calcd for $C_{23}H_{30}NO_7P$ MNa^+: 486, found: 486. ^1H NMR (400 MHz) δ 1.14 (3H, m), 1.47 (2H, m), 1.60 (2H, m), 1.70 (2H, m), 2.31 (2H, m), 3.17 (2H, m), 3.70 (3H, m), 4.75 (1H, s), 5.06 (2H, s), 6.97 (2H, d, $J=8.5$ Hz), 7.06 (2H, d, $J=8.5$ Hz), 7.30 (5H, m) ppm. ^{13}C NMR (100.6 MHz) δ 6.28, 17.50, 18.93, 21.89, 28.70, 33.52, 38.59, 52.75, 66.08, 72.98, 120.24, 121.3, 127.6 128.09, 128.43, 135.78, 136.70, 149.90, 172.29, 173.12 ppm.

5-{2-[4-(Ethyl-methoxy-phosphinoyloxy)-phenyl]-2-fluoro-acetylamino}-pentanoic acid benzyl ester (4). Diethylamino sulfur trifluoride (DAST, 184.7 μL, 1.4 mmol) was added to a solution of **1** (464 mg, 1 mmol) in CH_2Cl_2 (20 mL) at 0 °C. After stirring at 0 °C for 1 h, the solution was washed three times with brine and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography using a gradient of ethyl acetate to acetonitrile to afford 325.5 mg of **2** (70% yield). The silica gel was neutralized with triethylamine, washed with methanol and equilibrated with the eluent prior to chromatography. ESI-MS: calcd for $C_{23}H_{29}FNO_6P$ MNa^+: 488, found: 488. ^1H NMR (400 MHz) δ 1.21 (3H, m), 1.50 (2H, m), 1.60 (2H, m), 1.90 (2H, m), 2.40 (2H, m), 3.32 (2H, m), 3.80 (3 H, m), 5.12 (2H, s), 5.70 (2H, d, $J=48$ Hz), 7.20 (2H, d, $J=8.5$ Hz), 7.30 (5H, m), 7.40 (2H, d, $J=8.5$ Hz).

5-{2-[4-(Ethyl-methoxy-phosphinoyloxy)-phenyl]-2-fluoro-acetylamino}-pentanoic acid (5). Pd/C (10%, 3 mg) was added to a solution of **2** (30 mg, 0.065 mmol) in ethanol (10 mL). The solution was stirred under the hydrogen atmosphere for 1 h. The reaction mixture was then filtered through Celite and the solvent was removed under reduced pressure to afford 24 mg of pure **3** (99% yield). ESI-MS: calcd for $C_{16}H_{23}FNO_6P$ MH^+: 376.12, found: 376. ^1H NMR (400 MHz) δ 1.22 (3H, m), 1.50 (2H, m), 1.60 (2H, m), 1.89 (2H, m), 2.30 (2H, m), 3.20 (2H, m), 3.80 (3H, m), 5.70 (1H, d, $J=48$ Hz), 7.20 (2H, $J=8.5$ Hz), 7.40 (2H, $J=8.5$Hz) ppm. ^{13}C NMR (100.6 MHz) δ 6.29, 17.64, 19.07, 20.80, 21.72, 28.62, 33.30, 38.58, 53.05, 90.07, 91.95, 120.61, 128.20, and 168.42 ppm.

General procedure for the preparation of the *N*-hydroxysuccinimide esters 7 and 43

1,3-Dicyclohexylcarbodiimide (16.5 g, 0.08 mmol) was added to a solution of the corresponding acids (**5** and **42**, 0.04 mmol) and *N*-hydroxysuccinimide (9.2 mg, 0.08 mmol) in dioxane (5 mL). The solution was stirred at ambient temperature for 16 h. The urea was then removed by filtration and the solvent was evaporated. The crude compound was purified by flash chromatography using a gradient of ethyl acetate to acetonitrile. The products were isolated with 90% yield.

Compound 7. ESI-MS: calcd for $C_{20}H_{26}FN_2O_8P$ MNa^+ 495, found 495. ^1H NMR (400 MHz) δ 1.22 (3H, tt), 1.70 (2H, m), 1.79 (2H, m), 1.85 (2H, m), 1.91 (2H, m), 2.64 (2H, t), 2.85 (4H, s), 3.37 (2H, m), 5.72 (1H, d, $J=48$ Hz), 6.68 (2H, d), 7.23 (2H, d, $J=8.5$ Hz), 7.42 (2H, d, $J=8.5$ Hz) ppm. ^{13}C NMR (100.6 MHz): δ 6.43, 13.0, 17.78, 19.19, 21.78, 25.31, 25.31, 25.56, 28.40, 30.51, 38.42, 90.22, 92.09, 120.60, 128.30, 168.31, 169.18 ppm.

Compound 43. ^1H NMR (400 MHz) δ 1.23 (3H, m), 1.39 (2H, m), 1.54 (2H, m), 1.70 (2H, m), 2.30 (2H,m), 2.58 (3H, s), 2.64 (2H, m), 2.84 (4H, s), 3.28 (2H, m), 5.90 (2H, d, $J=48$ Hz), 7.18 (1H, d, $J=8.5$ Hz), 7.28 (2H,d, $J=8.5$ Hz) 7.32 (1H, d, $J_1=8.8$ Hz, $J_2=7.5$ Hz), 7.33 (1H, s), 7.67 (1H, d, $J=7.5$ Hz), 7.72 (1H, d, $J=8.8$ Hz) ppm.

3-Hydroxy-3-(4-hydroxy-phenyl)-propionic acid (39).[32] *n*-Butyl lithium (35.6 mmol, 23.7 mL 1.5 M in hexane) was added to a solution of diisopropylamine (5 mL, 35.6 mmol) in THF (45 mL). The mixture was stirred for 15 min at −78 °C. Benzyl acetate (5.94 mL, 39.1 mmol) was then added to the reaction mixture. After stirring for one h at −78 °C, 4-trimethylsiloxy-benzaldehyde (**37**, 6.9 g, 35.6 mmol) was also added. After 3 h at −78 °C, the reaction was quenched with NH_4Cl (saturated solution, 50 mL) and the pH adjusted between 5 to 6. The reaction mixture was extracted with ethyl acetate (3×50 mL) and the combined organic layers were dried over Na_2SO_4 and evaporated. The solid residue containing **38** was dissolved in 0.05% TFA in acetonitrile/methanol (1:1, 50 mL). After 12 h stirring at ambient temperature, the solvent was removed and the residue was dissolved in ethanol (25 mL). Pd/C (0.6 g, 10%) was carefully added to the reaction vessel. The mixture was stirred under hydrogen atmosphere (1 atm) for 3 h and filtered through Celite. The solvent was evaporated to afford 5.06g of **39** (yield 78%). The product (ESI-MS: calcd for $C_9H_{10}O_4$ MH^+ 183, found 183) was immediately used in the following reaction with no further purification.

5-[3-Hydroxy-3-(4-hydroxy-phenyl)-propionylamino]-pentanoic acid benzyl ester (40). 3-Dimethyamino propyl)-3-ethylcarbodiimide hydrochloride anhydrous (EDC, 1.18 g, 6.2 mmol) was added to a solution of **39** (1.04 g, 6.2 mmol), 1-hydroxybenzotriozle monohydrate (0.94 g, 6.2 mmol), diisopropylethylamine (1.99 g, 15.5 mmol) and 5-aminovaleric benzyl ester (2.95 g, 6.8 mmol) in DMF (50 mL). The solution was stirred at ambient temperature for 24 h. The DMF was then removed under reduced pressure and the residue was dissolved in ethyl acetate (50 mL). After washing with water, citric acid, and brine, the solution was dried (Na_2SO_4), the

crude compound was purified by flash column chromatography using a 1:1 mixture of ethyl ether/ethyl acetate as eluent to afford 1.67 g of **40** (55% yield). ESI-MS: calcd for $C_{21}H_{25}NO_5$ MH^+ 372, found 372. ^1H NMR (400 MHz) δ 1.47 (2H, m), 1.60 (2H, m), 2.36 (2H, s), 2.48 (2H, m), 3.23 (2H, m), 4.99 (1H, dd, $J_1=8.8$ Hz, $J_2=3.2$ Hz), 5.11 (2H, s), 6.76 (2H, d, $J=8.5$ Hz), 7.15 (2H, d, $J=8.5$ Hz), 7.33 (5 H, m) ppm. ^{13}C NMR (100.6 MHz) δ 21.96, 28.82, 33.66, 38.90, 39.11, 44.74, 66.37, 70.63, 115.45, 126.94, 128.26, 128.58,134.54, 135.77, 155.83, 172.06, 173.58 ppm.

5-(2-{4-[(3-Acetyl-phenoxy)-ethyl-phosphinoylmethyl]-phenyl}-2-hydroxy-acetylamino)-pentanoic acid benzyl ester (41). Diisopropylethylamine (260 mg, 2 mmol) was added to a solution of ethyl phosphonate dichloride (146.9 mg, 1 mmol) in CH_2Cl_2 (5 mL). 2-Hydroxyacetophenone (152.15 mg, 1 mmol) was added and the solution was stirred at ambient temperature for 1 h. **40** (357.16 mg, 1 mmol) was added followed by diisopropylethylamine (195 mg, 1.50 mmol). After stirring for 3 h at ambient temperature, the reaction mixture was washed with brine and the solvents removed under reduced pressure. The residue was purified by flash column chromatography using a stepwise gradient strarting with ethyl ether/ethyl acetate and ending with ethyl acetate/acetonitrile. Purification afforded 290.6 mg of product (50% yield). ESI-MS: calcd for $C_{31}H_{36}NO_8P$ MNa^+ 604, found 604. ^1H NMR (400 MHz) δ 1.24 (3H, m), 1.41 (2H, m), 1.54 (2H, m), 1.98 (2H, m), 2.28 (2H, t), 2.40 (2H, m), 2.46 (3H, s), 3.14 (2H, m), 4.93 (1H, m), 5.02 (2H, s), 6.28 (1H, d, $J=5.7$ Hz), 7.05 (2H, d, $J=8.5$ Hz), 7.20 (2H, d, $J=8.5$ Hz), 7.30 (5H, m), 7.34 (1H, dd, $J_1=8.8$ Hz, $J_2=7.5$ Hz), 7.36 (1H, s), 7.64 (1H, d, $J=7.5$ Hz), 7.51 (1H, d, $J=8.8$ Hz) ppm. ^{13}C NMR (100.6 MHz) δ 6.48, 18.54, 19.95, 21.90, 26.68, 28.68, 33.57, 38.80, 44.44, 66.22, 70.16, 120.07, 120.48, 125.1, 125.30, 127.10, 128.21, 128.52, 130.0, 135.70, 138.70, 140.49, 149.30, 150.05, 171.72, 173.28 ppm.

5-(3-{4-[(3-Acetyl-phenoxy)-ethyl-phosphinoyloxy]-phenyl}-3-hydroxy-propionylamino)-pentanoic acid (42). Pd/C (10%, 3 mg) was added to a solution of **41** (25 mg, 0.043 mmol) in ethyl acetate (5 mL). The solution was stirred under hydrogen atmosphere for 40 min and monitored by TLC. The reaction mixture was then filtered through Celite and the solvent was removed under reduced pressure to afford 21 mg of pure **42** (99% yield). ESI-MS: calcd for $C_{24}H_{30}NO_8P$ MH^+ 492, found 492. ^1H NMR (400 MHz) δ 1.28 (3H, m), 1.42 (2H, m), 1.51 (2H, m), 2.09 (2H, m), 2.25 (2H, m), 2.49 (2H, m), 2.60 (3H, s), 5.01 (1H, m), 6.76 (1H, d, $J=8.5$ Hz), 7.08 (2H, d, $J=8.5$ Hz), 7.27 (2H, d, $J=8.4$ Hz), 7.40 (2H, dd, $J_1=8.40$ Hz, $J_2=7.0$ Hz), 7.60 (2H, s), 7.73 (2H, d, $J=7.0$ Hz) ppm. ^{13}C NMR (100.6 MHz) δ 6.45, 18.51, 19.92, 21.90, 26.7, 28.60, 33.40, 38.85, 44.70, 47.78, 70.17, 92.00, 120.14, 120.50, 125.30, 127.16, 130.15, 138.70, 171.96, 176.66, 191.40, 197.34 ppm.

5-(3-{4-[(3-Acetyl-phenoxy)-ethyl-phosphinoyloxy]-phenyl}-3-fluoro-propionylamino)-pentanoic acid 2,5-dioxo-pyrrolidin-1-yl ester (44). Diethylamino sulfur trifluoride (DAST, 6.6 μL, 0.05 mmol) was added to a solution of **43** (10 mg, 0.017 mmol) in CH_2Cl_2 (20 mL) at −78 °C. After stirring at that temperature for 3 h the solution was washed three times with brine and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography using a gradient of ethyl acetate to acetonitrile to afford 3 mg of **44** (30% yield). The silica gel was neutralized with triethylamine, washed with methanol and equilibrated with the eluent prior to chromatography. ESI-MS: calcd for $C_{28}H_{32}FN_2O_9P$ MH^+ 591, found 591. ^1H NMR (400 MHz) δ 1.22 (3H, m), 1.38 (2H, m), 1.54 (2H, m), 1.70 (2H, m), 2.15 (2H, m), 2.58 (3H, s), 2.64 (2H, m), 2.84 (4H, s), 3.28 (2H, m), 5.90 (1H, d, $J=48.0$ Hz), 6.78 (2H, d, $J=8.5$ Hz), 7.18 (1H, dd, $J_1=7.5$ Hz, $J_2=8.8$ Hz), 7.20 (1H, d, $J=8.5$Hz), 7.32 (1H, m, $J_1=8.8$ Hz, $J_2=7.5$Hz), 7.33 (1H, s), 7.67 (1H, d, $J=8.5$ Hz), 7.72 (1H, d, $J=8.8$ Hz) ppm.

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