Acta Chemica Scandinavica, 1999: 53: 473–479
Printed in UK. All rights reserved

ACTA
CHEMICA SCANDINAVICA
ISSN 0904 213X

Hydrolysis of Organophosphorus Nerve Agent Soman by the Monoclonal Antibodies Elicited Against an Oxyphosphorane Hapten

Jari Yli-Kauhaluoma,[a,*] Tarmo Humppi[b] and Ari Yliniemelä[a,†]

[a]VTT Technical Research Centre of Finland, Chemical Technology, Catalytic Synthesis Technology, Biologinkuja 7, FIN-02150 Espoo, Finland and [b]Defence Forces Research Centre, NBC Defence Laboratory, PO Box 5, FIN-34111 Lakiala, Finland

Yli-Kauhaluoma, J., Humppi, T. and Yliniemelä, A., 1999. Hydrolysis of Organophosphorus Nerve Agent Soman by the Monoclonal Antibodies Elicited Against an Oxyphosphorane Hapten. – Acta Chem. Scand. 53: 473–479. © Acta Chemica Scandinavica 1999.

The antibody-mediated hydrolysis of the nerve agent *O*-1,2,2-trimethylpropyl methylphosphonofluoridate (soman) **1** has now been established with two monoclonal antibodies raised against the cyclic pentacovalent methyloxyphosphorane hapten **10** that mimics the pentacoordinated trigonal bipyramidal transition-state of the reaction. The hydrolysis reaction was studied using molecular orbital methods at the MP2/6-31+G*//HF/6-31+G* level of accuracy. According to the *ab initio* calculations, the reaction seems to proceed via three separate transition-states. The calculations are in good agreement with the experimental results. The 1,3-dioxabenzophosphole hapten **10** was synthesized, coupled to the carrier protein and the antibodies were obtained by the hybridoma technique. Two antibodies, DB-108P and DB-108Q were found to enhance the rate of soman hydrolysis and they were kinetically characterised.

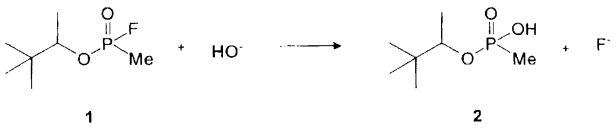
The nerve agent soman, *O*-1,2,2-trimethylpropyl methylphosphonofluoridate **1**, is extremely toxic as it potently inactivates the acetylcholinesterase enzyme (EC 3.1.1.7; AChE) by binding to its active site and irreversibly phosphorylating the active serine residue.[1] Thus, the function of the neurotransmitter acetylcholine is blocked and this may have a lethal outcome unless effective medical treatment is given immediately after intoxication.[2]

Despite an international agreement on disarmament of chemical warfare agents, the stockpiles of the organophosphorus nerve agents can still be regarded as a significant environmental and toxicological problem.[3] Moreover, organophosphates such as paraoxon, parathion and malathion are commonly used as insecticides in agriculture, and poisoning may occur in humans (or other mammals) following accidental exposure. Taken together, it is evident that there is a need for new methods of prophylaxis and treatment for organophosphate intoxication. Soman inactivation is particularly well suited for a catalytic antibody approach as the catalytic antibody would not only stochiometrically bind soman *in vivo*, but also convert it into relatively non-toxic products and release them, regenerating the catalytic activity of the antibody.

Antibodies catalysing particular reactions have been obtained by eliciting an immune response to favourably designed analogues of the transition-state of a reaction.[4] The antibodies raised against the transition-state analogues are expected to be capable of recognising, binding and stabilising the actual transition-state and to accelerate the reaction. In fact, the transition-state stabilisation is one of the major mechanisms of the enzyme catalysis of various biochemical reactions.

It has been suggested that catalytic antibodies could be used in the decomposition of organophosphates by raising them against the transition-state analogues of the corresponding hydrolysis reactions.[5] Indeed, there are currently four examples of antibody-catalysed hydrolysis reactions of organophosphates: the hydrolysis of a phosphate 4-nitrophenyl monoester,[6] the nerve agent soman,[7] a cyclic phosphate triester[8] and the insecticide paraoxon.[9] These antibodies with 'organophosphatase' activity were elicited against haptens that include an α-hydroxyphosphonate,[6] a quaternary amine[8] or an amine oxide[8,9] moiety as a transition-state mimic of the organophosphate hydrolysis. α-Hydroxyphosphonate was used to mimic a species that is involved in the attack of water on the phosphate monoester.[6] The charged *N*-oxide

* To whom correspondence should be addressed.
† Present address: Pharmacy of Korso, Maakotkantie 6, FIN-01450 Vantaa, Finland.



1 2

Scheme 1.

haptens and quaternary amine antigens were expected to elicit antibodies that catalyse the hydrolysis reactions by either stabilising a polar transition-state or inducing a reactive amino acid side chain that may act as a general base inside the antibody's binding pocket.[8,9]

The hydrolysis of soman affords the relatively non-toxic *O*-1,2,2-trimethylpropyl methylphosphonic acid **2** (Scheme 1). The rate of the hydrolysis reaction is pH-dependent, being faster at low and high pH. Soman is most stable in its aqueous solutions in the pH range 4–6. The rate of the soman hydrolysis is at its lowest at pH 4, where the half-life ($t_{1/2}$) is 250 h at 30 °C.[10] The hydrolysis of *O*-alkyl alkylphosphonofluoridates is thought to proceed via an in-line displacement mechanism through a pentacoordinated trigonal bipyramidal transition-state with the attacking nucleophile (hydroxide) and leaving group (fluoride) in the apical positions.[11]

Aiming at promotion and control of hydrolysis of the organophosphorus nerve agent soman with catalytic monoclonal antibodies, we considered the use of methyloxyphosphorane **10** as the geometric mimic of a transition-state of the soman hydrolysis as previously shown by Brimfield *et al.*[7] We also wanted to study the hydrolysis of soman with *ab initio* methods to characterise the transition-state of the reaction using *O*-methyl methylphosphonofluoridate as a simplified surrogate of soman. With an accurate knowledge of the transition-state structure, the comparison between transition-state and its phosphorane analogue **10** can be made, with the ultimate purpose of generating catalytic antibodies against the transition-state analogue.

This paper describes the synthesis of a transition-state analogue **10**, a theoretical study of the hydrolysis reaction of *O*-methyl methylphosphonofluoridate using *ab initio* molecular orbital methods, the coupling of **10** to a carrier protein, the production of monoclonal antibodies against **10**, screening of the antibodies for catalytic activity and also the determination of kinetic parameters of the found catalysts.

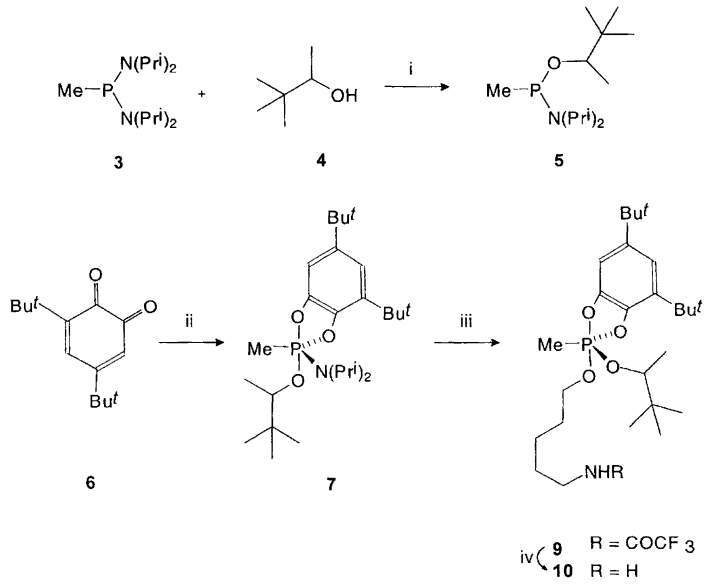
Results and discussion

Synthesis of the transition-state analogue. Our aim was to synthesize a transition-state analogue that has an aliphatic primary amino group to serve as the point of attachment for the link to the carrier protein, keyhole limpet hemocyanin. We envisage the synthesis of the 2-(5-aminopentoxy)-2-[(1,2,2-trimethyl)propoxy]-2-methyl-(4,6-di-*tert*-butyl)-2-benzophosphole **10** by analogy with Moriarty *et al.*,[12] with the exception of using the 5-amino group of the attached aminopentyl moiety rather than that of the 4-amino-3,3-dimethyl-2-butyl moiety of Moriarty's hapten as a coupling point to the carrier protein. It has been demonstrated in several instances that the failure of designed transition-state analogues to elicit effective catalytically active antibodies may be due to the location of the tethering site of the transition-state analogue to its carrier protein.[13] Therefore, we embarked on the preparation of phosphorane hapten **10** with a different tether site for its attachment to the carrier protein hoping that this would expose unique epitopes for recognition during the immunisation.

The synthesis of **10** started with methylation of bis(diisopropylamino)chlorophosphine with methyllithium in diethyl ether to give the diaminophosphine[12] **3** and the subsequent displacement reaction with (±)-3,3-dimethyl-2-butanol **4** and 1*H*-tetrazole as a nucleophilic catalyst to afford diisopropylamino(methyl)-(1,2,2-trimethylpropyloxy)phosphine **5** in 77% yield (Scheme 2). The diastereomeric mixture of aminomethylphosphine **5** was allowed to react with 3,5-di-*tert*-butylbenzoquinone[14] **6** to afford the unstable aminophosphorane **7** that was not isolated. The aminophosphorane **7** was immediately allowed to react with 5-trifluoroacetamidopentanol **8** at room temperature to furnish phosphorane **9** (≈1 : 1 mixture of diastereomers) in 87% yield. 5-(Trifluoroacetamido)pentanol **8** was in turn prepared using 1,1,3,3-tetramethylguanidine to catalyse amide formation between methyl trifluoroacetate and 5-aminopentanol. This reaction afforded **8** in 98% yield. The trifluoroacetyl group was chosen as a temporary protective group as it is easily removable in very mild basic conditions and as the phosphoranes are susceptible to hydrolysis in the presence of acid and in strongly basic conditions.[12] With phosphorane **9** in hand, its trifluoroacetyl group was removed by mild basic hydrolysis (0.1 M aq. NaOH, 4 °C, 1 h) to give the phosphole hapten **10** in 91% yield.

Attachment of the transition-state analogue **10** *to a carrier protein and the production of monoclonal antibodies.* The diastereomeric mixture of phosphorane hapten **10** was coupled to the free ε-amino groups of keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) with glutaraldehyde, which is a bifunctional cross-linking agent.[15] Immunoconjugates were purified by gel filtration and their purity and homogeneity were checked by sodium dodecyl sulphate–polyacrylamide gel electrophoresis[16] (SDS-PAGE) with Coomassie blue staining prior to the immunisation.

The immunoconjugate **10**–KLH was used to immunise BALB/c mice for the production of monoclonal antibodies according to the standard protocols.[17] Nine monoclonal antibodies were shown by an enzyme-linked immunosorbent assay[18] (ELISA) to bind to the phosphorane hapten **10** conjugated to bovine serum albumin.



Scheme 2. Reagents, solvents and yields: i, 1*H*-tetrazole, CH_2Cl_2, (90%); ii, **5**, CH_2Cl_2 (not isolated); iii, **8** (87%); iv, NaOH, H_2O (91%).

All nine monoclonal antibodies from culture media were of the IgG isotype and were purified by ammonium sulphate precipitation, anion exchange (DEAE), cation exchange (mono Q) and affinity chromatography (protein G). Antibodies were judged to be >95% homogeneous by SDS-PAGE with Coomassie blue staining.

Computational chemistry: hydrolysis. The reaction of *O*-methyl methylphosphonofluoridate (used as a representative organophosphorus model compound) and hydroxide ion was studied with *ab initio* methods. The HF/6-31+G* level gas phase reaction calculations indicate a multistep reaction profile (Fig. 1). The reaction energetics are given in Table 1. The reaction proceeds via three separate transition-states (S2, S4 and S6). While the transition-states S2 and S6 are highly asymmetrical with respect to the attacking hydroxide and the eliminating fluoride, the stationary points S3, S4 and S5 are almost identical. The transition-state S4 corresponds to the rotation of the methoxy group, coupled with slight changes of the P–OH and P–F distances (S3: P–OH = 1.75 Å, P–F = 1.71 Å, S5: P–OH = 1.72 Å, P–F = 1.78 Å). Under basic conditions, the reaction is known to proceed without any added catalyst. The very small activation energy for the first reaction step (S1–S3) and the exothermicity of the overall reaction are in good

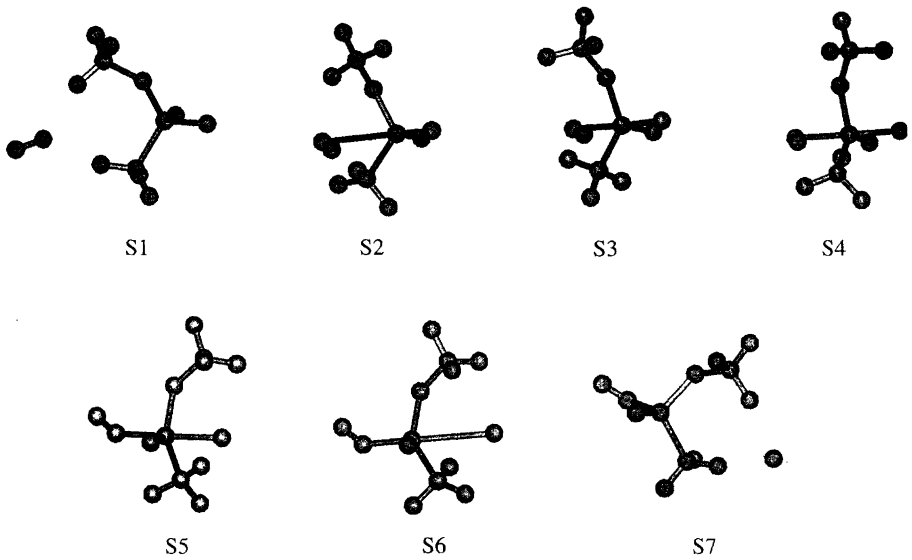


Fig. 1. The hydrolysis of *O*-methyl methylphosphonofluoridate. The geometry optimisations were carried out at the HF/6-31+G* level of accuracy.

Table 1. The energetics of the *O*-methylphosphonofluoridate hydrolysis, in kcal mol^{-1}. The structure numbering corresponds to Fig. 1.

Structure	S1	S2	S3	S4	S5	S6	S7
RHF/6-31+G*	0.000	3.654	−14.179	−11.682	−15.639	−9.645	−16.542
RHF/6-31+G*[a]	0.000	3.969	−12.000	−9.952	−13.478	−8.116	−15.116
MP2/6-31+G*//RHF/6-31+G*	0.000	2.401	−15.670	−12.895	−17.291	−9.609	−16.117
MP2/6-31+G*//RHF/6-31+G*[a]	0.000	2.715	−13.492	−11.165	−15.114	−8.080	−14.693
Imaginary frequency (cm^{-1})		126.36		100.77		105.32	

[a]Corrected for HF/6-31G* zero point vibrational energy.

agreement with the experimental results. The activation barriers suggest at least some stability for the intermediate S5.

Computational chemistry: hapten. An HF/6-31G* level optimised model of the phosphorane hapten **10** was compared with the reaction transition-states S2, S4, and S6. In Fig. 2, the heavy atoms of the transition-state S2 (possessing the highest energy) are superimposed on the respective atoms of the hapten. The RMS deviation between S2 and the hapten is 0.51 Å. The hapten and the transition-state S4 had an RMS deviation of 0.29 Å, while the RMS deviation with respect to the transition-state S6 is 0.51 Å. Thus, all three transition-states are similar to the hapten and it is difficult to say which transition-state is stabilised most by the catalytic antibody. However, provided that the antibody does not affect the overall reaction mechanism, the catalytic antibody obviously stabilises all three transition-states with respect to the reactants and products.

Kinetic characterisation of the antibodies. Two of the nine monoclonal antibodies to the oxyphosphorane hapten **10** exhibited soman hydrolysing properties as evidenced by a decrease in the inhibition of AChE enzyme. The degree of AChE inhibition is directly proportional to the concentration of the unhydrolysed soman in the presence of monoclonal antibodies using Ellman's procedure.[19] Two antibodies to **10**, DB-108P and DB-108Q, were catalytic and their kinetic behaviour was studied in more detail using gas chromatography–mass spectrometry (GC–MS) assay. Under the reaction conditions, DB-108P and DB-108Q catalysed the hydrolysis of **1** in a manner consistent with Michaelis–Menten kinetics (Table 2). A Lineweaver–Burk plot of the steady-state data was constructed to afford the values of k_{cat} and K_M. The observed first-order rate constant for the uncatalysed hydrolysis of **1** in the same buffer system was determined to be 4.5×10^{-4} min^{-1}. Antibody-catalysed hydrolysis reactions could be saturated by soman and displayed multiple turnovers. We also wanted to investigate whether the immunoglobulins DB-108P and DB-108Q were able to accelerate the hydrolysis of *O*-1,2,2-trimethylpropyl methylphosphonochloridate (chlorosoman). Unfortunately, even the background rate of chlorosoman hydrolysis was too fast under the reaction conditions studied.

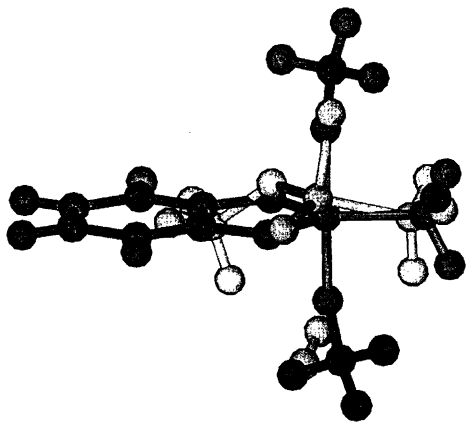


Fig. 2. The transition state S2 (light grey) superimposed on the HF/6-31G*-optimised model of the hapten (dark grey).

In summary, we have shown that immunisation with the trigonal, bipyramidal pentacoordinated phosphorane hapten **10** is able to elicit monoclonal antibodies that accelerate the hydrolysis rate of the highly toxic acetylcholinesterase (AChE) inhibitor soman **1**. These data also confirm the observations by Brimfield *et al.*[7] Despite the rather modest ability of the antibodies DB-108P and DB-108Q to catalyse the hydrolysis of soman, these antibodies may also have potential value in detoxificating organophosphates *in vivo*. The use of anti-soman antibodies to recognise, bind, immobilise and finally inactivate the toxin in an affinity-based treatment has previously been investigated by raising polyclonal and monoclonal antibodies against soman.[20] Despite their ability to compete with AChE for soman *in vitro*, these antibodies have failed to protect animals against soman intoxication when they are used in passive immunisation, mostly due to their low affinity.[21] Specific antibodies with hydrolytic properties could be used to decompose soman to nearly harmless products instead of just stoichiometrically immobilising it. One can also envisage an active immunis-

Table 2. Kinetic parameters for the hydrolysis of soman by the monoclonal antibodies DB-108P and DB-108Q.

Antibody	$V_{max}/10^{-2}$ μM min^{-1}	K_M/μm	$k_{cat}/10^{-2}$ min^{-1}	k_{cat}/k_{uncat}
DB-108Q	1.6	110	2.2	49
DB-108P	5.3	100	7.0	160

ation protocol with the appropriate transition-state analogue as a hapten in the prevention of organophosphate intoxications. These aspects and the improvement of the catalytic activity of the found 'somanase' antibodies by using site-directed mutagenesis and phage-display libraries will be the subject of further studies.

Experimental

Computational chemistry. The soman hydrolysis and the three-dimensional structure of the hapten were studied using molecular orbital methods. *O*-methyl methylphosphonofluoridate was used as a representative organophosporus model compound. The Spartan 4.0 program[22] package was used for preliminary visual studies. The Gaussian 94 program[23] package was used for the *ab initio* calculations. The reaction optimisations were carried out at the RHF/6-31+G* level of accuracy. The hapten was optimised at the HF/6-31G* level. All stationary points were fully optimised. The nature of each stationary point was assessed via vibrational frequency calculations. Second-order perturbation theory according to Møller and Plesset (MP2) was used for the electron correlation corrections.[24] The Insight II program package was used for the visual inspection of the computational results.[25]

Preparation of the hapten: General. Unless otherwise stated, reactions were carried out in oven-dried glassware under a positive atmosphere of argon or nitrogen. Reagents were transferred with plastic syringes and oven-dried or disposable needles. Dichloromethane was continuously distilled from calcium hydride. Anhydrous diethyl ether was distilled from sodium. All reagents were purchased from Aldrich (Weinheim, Germany), Sigma (St. Louis, Mo. USA) and Fluka (Buchs, Switzerland) and were used as supplied. All chromatography solvents of *purum* grade were obtained commercially and used without further purification. Bis(diisopropylamino)-(methyl)phosphine **3** was prepared from bis(diisopropylamino)chlorophosphine as described by Moriarty *et al.*[12]

Acetylcholinesterase (EC 3.1.1.7; AChE) from electric eel was obtained commercially (Sigma). Keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) were purchased from Fluka. Soman and soman-d_3 (>96% pure by GC–MS and ^{31}P NMR) were obtained from the NBC Defence Laboratory of the Defence Forces Research Centre (Lakiala, Finland).

The R_f values refer to the thin-layer chromatograms developed using 0.25 mm Merck silica gel 60 PF_{254} plates visualised with either ethanolic ninhydrin (1%), phosphomolybdic acid (5%), or an ultraviolet lamp. Yields are for unoptimised procedures and refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials, unless otherwise noted. All proton NMR spectra (600 MHz) were obtained in $CDCl_3$ at ambient temperature on a Varian-600 NMR instrument. Chemical shifts (δ) are reported in parts per million relative to internal reference tetramethylsilane and coupling constants (J) are given in Hz. Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br (broad). Electron ionisation and high and low resolution fast atom bombardment mass spectra were provided by VTT Mass Spectrometry Facility.

Diisopropylamino(methy)(1,2,2-trimethylpropoxy)phosphine **5**. A mixture of bis(dimethylamino)(methyl)phosphine **3** (4.0 g, 16 mmol), (±)-3,3-dimethyl-2-butanol **4** (2.1 cm^3, 17 mmol) and 1*H*-tetrazole (0.11 g, 1.6 mmol) in dichloromethane (100 cm^3) was stirred at room temperature for 2 days. The reaction mixture was evaporated *in vacuo* and the oily residue was extracted with pentane (2 × 30 cm^3) and filtered. The combined pentane extracts were evaporated *in vacuo* and the crude product was distilled to give **5** (6.3 g, 90%) as a colourless oily liquid; b.p. 54–56 °C at 130 Pa. δ_H (600 MHz; $CDCl_3$) 0.88 and 0.89 [9 H, 2 × s, $C(CH_3)_3$], 1.12 and 1.15 [6 H, 2 × d, $^3J_{HH}$ 6.2, $(CH_3)_2CH$], 1.13 (3 H, d, $^3J_{HH}$ 7.2, CH_3CHO), 1.19 and 1.21 [6 H, 2 × d, $^3J_{HH}$ 6.3, $(CH_3)_2CH$], 1.23 and 1.24 (3 H, 2 × d, $^2J_{HP}$ 6.5 and 6.6, CH_3P), 3.38 and 3.38 (1 H, 2 × q, $^3J_{HH}$ 6.6, CH_3CHO), 3.58 (2 H, m), 1:1 diastereomeric mixture. *m/z* (EI-MS) 247 (M^+, 11%), 232 (5), 163 (13), 148 (100), 146 (28), 120 (22), 106 (24) and 100 (8). *m/z* (FAB HRMS, 3-NBA) 247.2062 (M^+, $C_{13}H_{30}NOP$ requires 247.2065).

*2-(5-Trifluoracetamidopentoxy)-2-(1,2,2-trimethylpropoxy)-2-methyl-(4,6-di-*tert*-butyl)-1,3-dioxa-2λ^5-benzophosphole* **9**. A solution of **5** (1.0 g, 2.3 mmol) in anhydrous dichloromethane (30 cm^3) was added dropwise to a solution of 3,5-di-*tert*-butyl-1,2-benzoquinone **6** (0.53 g, 2.4 mmol) in anhydrous dichloromethane (20 cm^3) at 0 °C. The reaction mixture was stirred at 0 °C for 15 min and at room temperature for 2 h. The intermediate aminophosphorane **7** was not isolated but an EI-MS for **7** gave *m/z* (relative intensity) 467 (M^+, 1%), 384 (14), 300 (100), 282 (55), 267 (53), 211 (8) and 181 (12). The intermediate **7** was allowed to react with 5-(trifluoroacetamido)pentanol **8** (0.45 g, 2.3 mmol) at 0 °C for 1 h and at room temperature for 16 h. The reaction mixture was evaporated *in vacuo* and the crude product was purified by flash column chromatography, eluting with 10:2:1 *n*-hexanes–EtOAc–triethylamine to yield 2-(5-trifluoroacetamidopentoxy)-2-(1,2,2-trimethylpropoxy)-2-methyl-(4,6-di-*tert*-butyl)-1,3-dioxa-2λ^5-benzophosphole **9** (0.93 g, 87%) as a thick oil. δ_H(600 MHz; $CDCl_3$) 0.79 and 0.96 [9 H, 2 × s, $(CH_3)_3CHCH_3$], 1.09 (3 H, 2 × d, $^3J_{HH}$ 6.6, CH_3CHO), 1.31 and 1.32 [9 H, 2 × s, arom. $(CH_3)_3C$], 1.40 and 1.41 [9 H, 2 × s, arom. $(CH_3)_3C$], 1.43–1.54 [2 H, m, $O(CH_2)_2CH_2$], 1.63–1.73 (2 × 2 H, 2 × m, $^3J_{HH}$ 6.9, OCH_2CH_2 and $NHCH_2CH_2$), 1.82 and 1.85 (3 H, 2 × d, $^2J_{HP}$ 17.7, CH_3P), 3.43 (2 H, q, $^3J_{HH}$ 6.6, $CONHCH_2$), 3.79–3.93 (2 H, m, OCH_2), 4.14 and 4.37 (1 H, 2 × qd, $^3J_{HH}$ 6.6, CH_3CHO), 6.38 (1 H, br, s, CF_3CONH), 6.79 and 6.81 (1 H, 2 × s, arom. H), 6.82 and 6.83 (1 H, 2 × s, arom. H), 1:1 diastereomeric mixture. *m/z* (EI-MS) 565 (M^+, 4%), 523 (8), 509

(4), 395 (5), 368 (100), 282 (14) and 267 (95). *m/z* (FAB HRMS, 3-NBA) 565.3144 (M^+, $C_{28}H_{47}F_3NO_5P$ requires 565.3144).

5-(Trifluoroacetamido)pentanol **8**. A mixture of 5-aminopentanol (5.3 cm^3, 49 mmol), methyl trifluoroacetate (5.4 cm^3, 53 mmol) and 1,1,3,3-tetramethylguanidine (6.4 cm^3, 51 mmol) in dichloromethane (50 cm^3) was stirred at 5 °C for 1 h and at room temperature for 16 h. The reaction mixture was diluted with dichloromethane (100 cm^3) and the organic layer was washed with 0.1 M hydrochloric acid (75 cm^3) and brine (2 × 50 cm^3). The organic layer was dried with anhydrous Na_2SO_4, filtered and evaporated *in vacuo*. The crude product was purified by silica gel chromatography, eluting with 10% MeOH–EtOAc to afford **8** (9.5 g, 98%) as a thick oil. δ_H (600 MHz; $CDCl_3$) 1.43 [2 H, m, $^3J_{HH}$ 7.2, $^4J_{HH}$ 2.4, $HO(CH_2)_2CH_2$], 1.58 (2 H, m, $^3J_{HH}$ 6.6, $NHCH_2CH_2$), 1.62 (2 H, m, $^3J_{HH}$ 7.2, $HOCH_2CH_2$), 2.53 (1 H, br s, CH_2OH), 3.36 (2 H, q, $^3J_{HH}$ 7.2, $CONHCH_2$), 3.64 (2 H, q, $^3J_{HH}$ 6.6, CH_2OH), 7.33 (1 H, br s, CF_3CONH). *m/z* (FAB LRMS, 3-NBA) 200 ($M^+ + 1$).

*2-(5-Aminopentoxy)-2-(1,2,2-trimethylpropoxy)-2-methyl-(4,6-di-*tert*-butyl)-1,3-dioxa-2λ⁵-benzophosphole* **10**. A solution of trifluoroacetamide **9** (0.20 g, 0.43 mmol) in aqueous 0.1 M sodium hydroxide solution (22 cm^3, 2.2 mmol) was stirred at 4 °C for 1 h. The reaction mixture was diluted with cold brine (50 cm^3) and extracted with ethyl acetate (3 × 75 cm^3). The combined organic layers were washed with brine (40 cm^3), dried with Na_2SO_4 and evaporated *in vacuo* to give the crude product that after purification by flash column chromatography on silica, and elution with 5% EtOAc–triethylamine afforded 2-(5-aminopentoxy)-2-(1,2,2-trimethylpropoxy)-2-methyl-(4,6-di-*tert*-butyl)-1,3-dioxa-2λ⁵-benzophosphole **10** (0.18 g, 91%) as a colourless, thick liquid. δ_H (600 MHz, $CDCl_3$) 0.84 and 0.95 [9 H, 2 × s, $(CH_3)_3CH$], 1.29 (3 H, d, $^3J_{HH}$ 6.5, CH_3CHO), 1.30 and 1.31 [9 H, 2 × s, arom. $(CH_3)_3C$], 1.44 and 1.45 [9 H, 2 × s, arom. $(CH_3)_3C$], 1.46–1.51 [2 H, m, $O(CH_2)_2CH_2$], 1.62–1.71 (4 H, m, OCH_2CH_2 and $NHCH_2CH_2$), 1.71 (3 H, d, $^2J_{HP}$ 18, CH_3P), 3.42 (2 H, q, $^3J_{HH}$ 6.9, CH_2NH_2), 3.71 (2 H, t, $^3J_{HH}$ 6.3, CH_2O), 4.16 (2 H, q, $^3J_{HH}$ 6.9, CH_2NH_2), 4.42 (1 H, m, CH_3CHO), 6.95 (1 H, s, arom. H), 7.14 (1 H, s, arom. H), 1:1 diastereomeric mixture. *m/z* (FAB LRMS, 3-NBA) 470 ($M^+ + 1$). *m/z* (FAB HRMS, 3-NBA) 469.3320 (M^+, $C_{26}H_{48}NO_4P$ requires 469.3321).

Preparation of the immunoconjugate. The antigen **10** (6 mg, 13 μmol) dissolved in *N,N*-dimethylformamide (0.16 cm^3) was added to 1.28 cm^3 of a solution of KLH or BSA (4 mg) in phosphate buffer (50 mmol dm^{-3}, pH 7.4). A solution of glutaraldehyde in water (0.16 cm^{-3}, 20 v/v%) was added with stirring at room temperature. After 24 h at room temperature, the immunoconjugates **10**–keyhole limpet hemocyanin (KLH) and **10**–bovine serum albumin (BSA) were purified by gel filtration. Their purity and homogeneity was checked by sodium dodecyl sulphate–polyacrylamide gel electrophoresis[16] (SDS-PAGE) with Coomassie blue staining prior to the immunisation. Protein conjugates were stored at −18 °C prior to use.

Monoclonal antibodies. Monoclonal *anti*-**10** antibodies from mice immunised with **10**–KLH conjugates were prepared by DiaBor Ltd. (Oulu, Finland), using the standard protocols.[17] Briefly, five six-week old female BALB/c mice were immunised in three week intervals with **10**–KLH (35 μg antigen/mouse) emulsified in Freund's adjuvant (Difco Laboratories, USA). The first injection was given in complete adjuvant subcutaneously, the first booster in incomplete adjuvant intraperitoneally and the second booster intavenously. Serum samples were tested after the second booster and the mouse showing best response against the **10**–BSA antigen in direct ELISA was selected for fusion. The selected mouse received an intravenous booster of antigen three days before the fusion. Spleen cells from the immunised animal were fused with mouse myeloma cell line (P3-X63-Ag8.653) using polyethylene glycol Gibco PEG 4000 (Gibco, United Kingdom). Hybrid cells were selected on 96-well plates in HAT medium [DMEM high glucose (Gibco, UK) supplemented with 10% NCTC-135 (Gibco, UK), 10% CPSR serum (Sigma, USA), 10% fetal bovine serum (Bioclear, UK), 5% HECS (Costar, The Netherlands), HAT supplement 50X (Gibco, UK) and Penicillin/Streptomycin Solution 100X (Gibco, UK)]. Hybrid cells were screened using direct ELISA against the antigen. Medium from nine positive hybrids was collected and the monoclonal antibody purified by ammonium sulphate precipitation, anion exchange chromatography (DEAE-Sephacel), cation exchange chromatography on a mono Q column and finally affinity purification on a protein G-Sepharose column (Pharmacia, Sweden). Antibodies were judged to be >95% homogeneous by SDS-PAGE with Coomassie blue staining.

Direct ELISA in the production of monoclonal antibodies. 96-Well EIA plates (Dynatech, USA) were coated with 400 ng/well of **10**–BSA antigen in PBS (10 mol dm^{-3} potassium phosphate, 150 mmol dm^{-3} NaCl, pH 7.4) overnight at 4 °C. Plates were washed (Anthos AutoWash, UK) four times with PBS and blocked with 1% BSA in PBS for 30 min. Samples were incubated for 60 min and then washed with PBS, after which the bound antibody was detected using anti-mouse Vector ABC kit (Vector Laboratories, USA) and ABTS substrate kit for horseradish peroxidase (Vector Laboratories, USA) according to the manufacturer's instructions. Plates were read at 405 nm (Anthos 2001, UK) using the MultiCalc program (Pharmacia, Sweden).

Screening of the antibodies for catalytic activity. Catalytic properties of the nine monoclonal antibodies were deter-

mined by measuring the degree of the inhibition of acetylcholinesterase (AChE) by unhydrolysed soman **1** present in the reaction mixtures. The concentration of inactivated AChE is directly proportional to the concentration of soman present in the reaction mixtures. The enzymatic activity was determined employing Ellman's method used for quantitative detection of thiol groups in thiocholine released during the hydrolysis of AChE.[19] Thiol reacts with bis(3-carboxy-4-nitrophenyl) disulphide producing the yellow 2-nitro-5-thiobenzoate anion. (**CAUTION!** As soman is an extremely toxic AChE inhibitor, concentrations of soman higher than 1 mM were not used.) 96-Well plates were used for the hydrolysis reactions and the subsequent spectrophotometric determinations of the residual AChE activity. The ratios $\Delta A/\Delta A_0$ were determined (ΔA and ΔA_0 are the changes in absorbance/min in the presence and absence of the monoclonal antibody, respectively). $\Delta A/\Delta A_0$ are the changes in absorbance/min in the presence and absence of the monoclonal antibody, respectively). $\Delta A/\Delta A_0$ is >1 if the monoclonal antibody enhances the rate of soman hydrolysis. The incubation time of the reactions was 2 h.

Determination of the kinetic constants of the antibody catalysts. The kinetic parameters of the antibody catalysts found were determined by means of gas chromatography–mass spectrometry assay. Aliquots of the reactions were extracted with ethyl acetate, and the concentration of soman was determined using GC–MS. Deuteriated soman, *O*-1,2,2-trimethylpropyl methyl-d_3-phosphonofluoridate, was added as an internal standard for GC–MS assay in all the hydrolysis reactions. The concentration of the hydrolysis product *O*-1,2,2-trimethylpropyl methylphosphonic acid **2** was determined as the difference between the initial and final concentration of soman present in the reaction mixtures according to the stoichiometry of the hydrolysis reaction. For our assays we typcially set up 0.50 cm^3 reactions containing antibody DB-108P or DB-108Q (0.76 $\mu mol\ dm^{-3}$) in phosphate buffered saline, PBS (10 $mmol\ dm^{-3}$, pH 7.4) at 25 °C and 400–1000 $\mu mol\ dm^{-3}$ soman in the same buffer. Background reactions of the soman hydrolysis were monitored in the absence of the monoclonal antibodies.

Acknowledgements. We thank Mrs. Anja Salakari and Mrs. Erja Kantola for excellent technical assistance, Tarja Nevanen M.Sc. for the purification of the immunoconjugates and MATINE, Helsinki, Finland (Scientific Committee for National Defence) for financial support.

References

1. Koelle, G. B. *Fundam. Appl. Toxicol. 1* (1981) 129.
2. Hirshberg, A. and Lerman, Y. *Fundam. Appl. Toxicol. 4S* (1984) 209.
3. Goldblatt, J. In: *SIPRI Yearbook 1987. World Armaments and Disarmaments*, SIPRI Oxford, University of Oxford, Oxford 1987, p. 385.
4. Lerner, R. A., Benkovic, S. J. and Schultz, P. G. *Science 252* (1991) 659; Schultz, P. G. *Acc. Chem. Res. 22* (1989) 287; Schultz, P. G. and Lerner, R. A. *Science 269* (1995) 1835.
5. Glikson, M., Arad-Yellin, R., Ghozi, M., Raveh, L., Green, B. S. and Eshhar, Z. *Mol. Immunol. 29* (1992) 903.
6. Scanlan, T. S., Prudent, J. R. and Schultz, P. G. *J. Am. Chem. Soc. 113* (1991) 9397.
7. Brimfield. A. A., Lenz, D. E. Maxwell, D. M. and Broomfield, C. A. *Chem. Biol. Interact. 87* (1993) 95.
8. Rosenblum, J. S., Lo, L.-C., Li, T., Janda, K. D. and Lerner, R. A. *Angew. Chem., Int. Ed. Engl. 34* (1995) 2275.
9. Lavey, B. J. and Janda, K. D. *J. Org. Chem. 61* (1996) 7633.
10. Franke, S. *Lehrbuch der Militärchemie 1*, Militärverlag der Deutschen Demokratischen Republik, Berlin 1977, pp. 95–102.
11. Knowles, J. R. *Ann. Rev. Biochem. 49* (1980) 877; Cox, J. R. and Ramsay, O. B. *Chem. Rev. 64* (1964) 317; Caldwell, S. R., Raushel, F. M., Weiss, P. M. and Cleland, W. W. *Biochemistry 30* (1991) 7444.
12. Moriarty, R. M., Hiratake, J. and Liu, K. *J. Am. Chem. Soc. 112* (1990) 8575; Moriatry, R. M., Liu, K., Tuladhar, S. M., Guo, L., Condeiu, C., Tao, A., Xu, W., Lenz, D. and Brimfield, A. *Phosphorus Sulfur Silicon 109–110* (1996) 237.
13. Yang, G., Chun, J., Arakawa-Uramoto, H., Wang, X., Gawinowicz, M. A., Zhao, K. and Landry, D. W. *J. Am. Chem. Soc.* 118 (1996) 5881.
14. Ramirez, F. and Desai, N. B. *J. Am. Chem. Soc. 85* (1963) 3252; Abdou, W. M., Mahran, M. R. H., Hafez, T. S. and Sidky, M. M. *Phosphorus Sulfur 27* (1986) 345.
15. Peters, K. and Richards, F. M. *Ann. Rev. Biochem. 46* (1977) 523.
16. Laemmli, V. *Nature (London) 227* (1970) 680.
17. Oi, V. T. and Herzenberger, L. A. In: Mishell, B. B. and Shiigi, S. M., Eds., *Selected Methods in Cellular Immunology*, Freeman, San Francisco 1980, pp. 351–372.
18. Engvall, E. *Methods Enzymol. 70* (1980) 419.
19. Ellman, G. L., Courtney, K. D., Andres, V. and Featherstone, R. M. *Biochem. Pharmacol. 7* (1961) 88.
20. Hunter, K. W., Lenz, D. E., Brimfield, A. A. and Naylor, J. A. *FEBS Lett. 149* (1982) 147; Heldman, E., Balan, A., Horowitz, O., Ben-Zion, S. and Torten, M. *FEBS Lett. 180* (1985) 243; Erhard, M. H., Kühlmann, R., Szinicz, L. and Lösch, U. *Arch. Toxicol. 64* (1990) 580.
21. Lenz, D. E., Brimfield, A. A., Hunter, K. W., Benschop, H. P., De Jong, L. P. A., Van Dijk, C. and Clow, T. R. *Fundam. Appl. Toxicol. 4S* (1984) 156.
22. Spartan 4.0 (Wavefunction Inc., Irvine, CA) on a Silicon Graphics Indigo II workstation was used for preliminary studies.
23. For the *ab initio* calculations Gaussian 94 [Frisch, M. J., Head-Gordon, M., Trucks, G. W., Foresman, J. B., Schlegel, H. B., Raghavachari, K., Robb, M. A., Binkley, J. S., Gonzales, C., Defrees, D. J., Fox, D. J., Whiteside, R. A., Seeger, R., Melius, C. F., Baker, J., Martin, R. L., Kahn, L. R., Stewart, J. J. P., Topiol, S. and Pople, J. A. Gaussian Inc., Pittsburgh, PA 1995] was used on a CRAY C94 supercomputer at CSC.
24. Møller, C. and Plesset, M. S. *Phys. Rev. 46* (1934) 618.
25. Insight II (Biosym Technologies, 9685 Scranton Road, San Diego, CA) on a Silicon Graphics Indigo II workstation was used for molecular graphics.

Received November 18, 1998.