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Immunologically driven antibodies chemical engineering: design and synthesis of a hapten aimed at nerve agent hydrolysis

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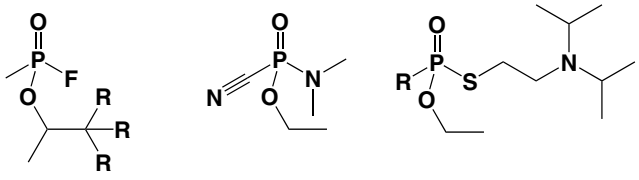
Abstract—In this letter, we describe the design and the synthesis of an organophosphorus hapten aimed at a mixed biotechnological–chemical strategy for the mild decontamination of exceedingly toxic nerve agent VX. Hapten will be used to raise and select monoclonal antibodies (mAbs) able to bind both the nerve agent, and an oxime, derived from pyridinaldoxime, able to specifically hydrolyze its P–S bond. In order to significantly increase the hydrolysis reaction rate, the controlled respective positioning of the oxime and of the substrate will be achieved through the immunization, and in a second step, the oxime will be bound to the mAb in a reactive position towards the reactive thiophosphate functionality of the substrate.

Acetylcholinesterase irreversible inhibitors such as organophosphorus derivatives Sarin (**1a**), Soman (**1b**), Tabun (**2**) or exceedingly toxic VX (**3a**) (Scheme 1) are nerve agents used as chemical warfare.[1,2] The international control of the proliferation of those weapons of mass destruction[3] is thwarted by the ease of their synthesis, and by the similarity between their chemical precursors and those of widely used pest-control agents.

The ease of production of these agents and their toxicity make them attractive to terrorist groups. The decontamination and demilitarization of organophosphorus nerve agents is therefore an urgent and topical problem. Most of the decontamination means intended at neutralizing those nerve agents use strong alkaline and/or oxidative media, and are thus incompatible with medical use, but also skin treatment or sensitive material.[4,5] In the last few years, after September 11, considerable amount of work has been undertaken on the biotechnological approaches to decontamination of these toxic substances.[6] In this letter, we want to display a dual biotechnological–chemical approach, aiming at an affordable and environmentally friendly process for decontamination of phosphonothioate VX.



Scheme 1. Nerve agents structures.

Among the nerve agents, VX, against which no satisfactory mild decontamination mean has been proposed yet, is by far the most difficult target to deal with, since (1) it is exceedingly toxic ($LD_{50} = 8$ μg/kg, i.v., and 28 μg/kg, percutaneous, rabbit),[5] (2) its hydrolysis can occur through at least four different pathways,[5,7] and (3) contrary to the phosphorus–sulfur bond cleavage, phosphorus–oxygen bond cleavage[8] gives rise to an equally toxic compound.[5] In order to have a less toxic VX mimic, but which displays equivalent hydrolysis features, we proposed the use of its aromatic equivalent PhX (**3b**) (Scheme 1).[9] Interestingly, PhX displays exactly the same hydrolysis profile than VX, but its toxicity has

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been estimated to be two orders of magnitude lower than VX, and in vitro AChE inhibition appears reversible.[10]

In order to find efficient biocompatible decontamination means against VX, we stressed our efforts on catalytic antibodies[11–15] which can be produced in high quantities at very low costs. Our first efforts were led on monoclonal antibodies selected for their ability to bind transition state analogues.[10,16] Catalytic but insufficiently active antibodies were obtained, and our results led us to the conclusion that a more efficient solution would be to find a way to graft a residue capable of cleaving the P–S bond in the binding site of an antibody. With this goal in mind, we described a new strategy[17] based on chemical engineering of monoclonal antibodies in order to covalently attach a targeted catalytic residue in the binding pocket of an anti-PhX mAb. Our approach can be considered as the merger of two already described strategies for catalytic antibodies: the use of cofactors,[18–20] and the chemical introduction of a catalytic function close to the antibody binding site.[21,22] The hapten will be designed to include binding of a nucleophile as well as the substrate in a reactive relative position. Taking into account the limitations raised by a previous study,[17] the catalytic residue itself will be then covalently attached onto the selected monoclonal antibody binding pocket.

The main advantages of our strategy rely on the facts that (1) the monoclonal antibodies are screened and selected through their binding abilities, which can be done through well established techniques. (2) Any catalytic residue can be grafted onto the antibody binding site, widening thus the amount of catalytic functions available. The residue yet requires strong antigenic determinants in order to efficiently and specifically fit into the mAb binding pocket. (3) The relative position of the catalytic residue and the substrate are controlled by the very structure of the hapten itself. Thus, through binding to the antibody, both compounds will be in a reactive position, lowering the required entropy of the reaction, and orienting the reactivity towards the P–S bond cleavage.

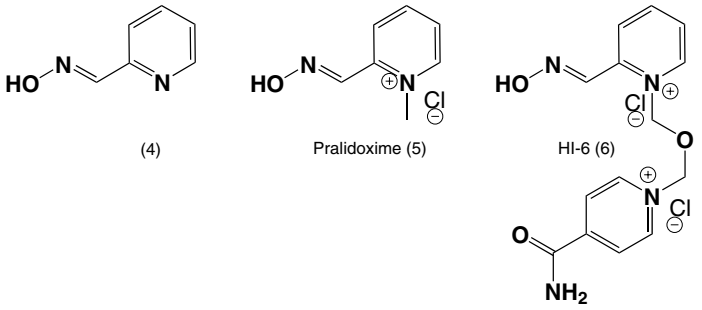
The first point to be solved is the selection of the suitable catalytic residue. With the selective P–S bond cleavage as a goal, two possibilities are available: a nucleophile displaying an α effect,[23,24] or a metallic species[25–28] able to activate a water molecule. For strategic reasons, the first solution was adopted. Among the α-nucleophiles, oximes[7,29] seemed to be the more suited since they cleave specifically the P–S bond,[30,31] and already shown great efficiency (including in vivo)[32,33] in reactivating poisoned cholinesterases. Other candidates such as peroxyacids,[34] peroxides[7,8,35] or iodosylcarboxylates,[36] are less adapted to an in vivo application, and might react with the mAbs. 2-Pyridinealdoxime (**4**), pralidoxime (**5**) and HI-6 (pyridinium, 1-[[[4-(aminocarbonyl) pyridinio]methoxy]methyl]-2-[(hydroxyimino)methyl]-, dichloride (**6**) (Scheme 2)[37,38] displaying both high P–S cleavage efficiency and antigenic determinants were chosen as structural bases for the catalytic residue. The turn-over for the catalyst will be achieved, since the intermediary nucleophilic addition product bears a particularly water labile P–O–N moiety. Synthetic dead ends dealing with the pyridinium based haptens rapidly led us to choose 2-pyridinaldoxime (**4**) as the target for the catalytic residue.

A first hapten (**H1**, Fig. 1) was thus designed, displaying the following features:

- A PhX analogue, with a noncleavable aminopropyl side chain, methylene replacing the sulfur atom in order to both lower the potential toxicity of this hapten, and to ensure the structural integrity of the hapten throughout the immunization procedure.
- An oxime analogue, mimicking the addition of the oxygen atom onto the phosphorus, opposite to the leaving group, in order to have a correct placement of the two partners to enhance the $S_N2(P)$ character of the reaction.[7]
- The labile P–O–N moiety is replaced by a non cleavable $P–CH_2–N$ motive.
- An aliphatic linker for hapten binding to carrier protein via homobifunctional reagents such as glutaraldehyde or disuccinimidyl suberate.

Hapten (**H1**) retrosynthetic scheme is relatively straightforward, with formation of the imine bond as the key step between aldehyde (**7**) and amine bearing phosphine oxide (**8**) (Scheme 3).

Aldehyde (**7**) (R_2 = phthalimide) was synthesized through monoformylation of 2,6-dibromopyridine, followed by introduction of the aminopropyl linker (Scheme 4). Monoformylation of 2,6-dibromopyridine



Scheme 2. Oxime structures.

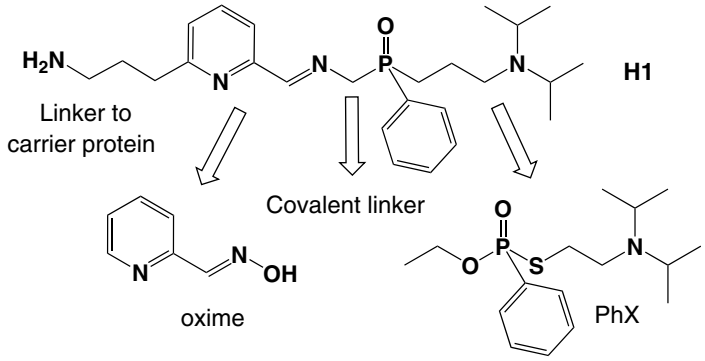
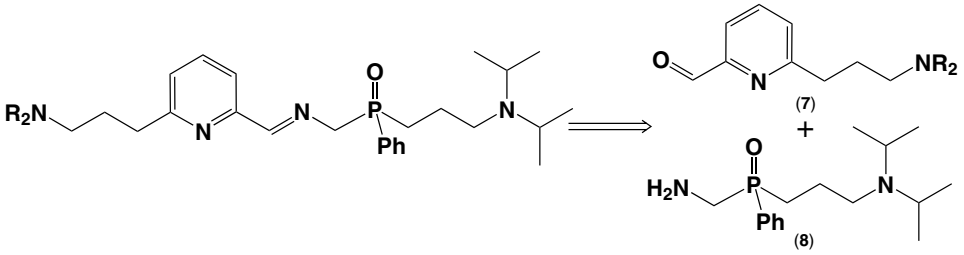


Figure 1. Structure of first hapten (**H1**).



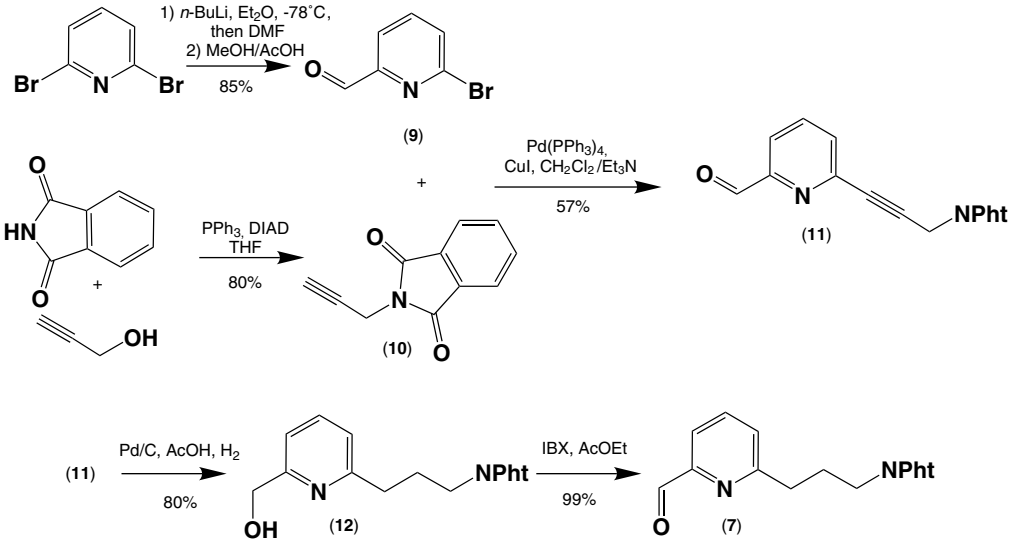
Scheme 3. Hapten (**H1**) retrosynthetic analysis.

was achieved via selective formation of 2-bromo-6-lithiopyridine (according to previously described procedures),[39] followed by electrophilic substitution with DMF. In our hands, best yields (85%) were obtained using diethyl ether as the solvent, with a careful monitoring of the temperature (−60 °C). Interestingly, the aliphatic linker could be readily introduced without protection of the formyl moiety through Sonogashira coupling between 6-bromo-pyridine-2-carbaldehyde and *N*-prop-2-ynyl phthalimide (**10**) (prepared from propargyl alcohol and phthalimide through Mistunobu reaction). Yields for the Sonogashira coupling is relatively modest (57%), but *N*-phthalimide moiety has already been described as poorly suitable for the Sonogashira coupling process.[40]

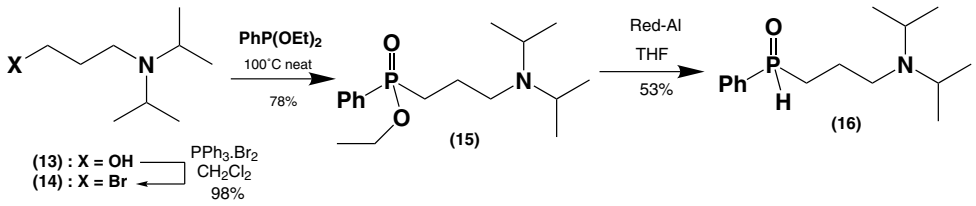
Clean hydrogenation of the triple bond was also a tricky task. When led using classical conditions, with platinum oxide, Raney Nickel or palladium as catalysts, only incomplete reduction of the alkyne to alkene was observed. Complete reduction to alkane was only achieved using higher hydrogen pressure, and resulted in the consecutive reduction of the conjugated aldehyde to the corresponding alcohol. Eventually, best and reproducible yields of alcohol (**12**) (76–80%) were obtained under drastic conditions: a concentrated 0.1 M solution of (**11**) in glacial acetic acid, with 12.5% molar Pd/C, and submitted to a 20 bar hydrogen pressure. Alcohol (**12**) was then readily oxidized to aldehyde (**7**) using IBX[41,42] with a nearly quantitative yield. Aldehyde (**7**) was thus synthesized in four steps and an overall 39% yield (Scheme 4).

Phosphine oxide (**8**) synthesis required attachment of both the tertiary aminopropyl and the aminomethylene side chains onto the phenyl phosphine oxide moiety. First attempts to bind the aminopropyl side chain onto the phosphorus atom through formation of organometallic species showed that the hindered tertiary amine moiety was poorly compatible even with Grignard or organozinc reagent. This was achieved through two successive Michaelis–Arbuzov reactions. The bromo amine (**14**) was formed from the corresponding alcohol (**13**)[43] through usual procedure (PPh_3; Br_2 in dichloromethane, 98% yield). Heating at 100 °C for 2 days a neat mixture of *O*,*O*-diethyl phenyl phosphonite and bromo amine (**14**) gave a satisfactory 78% yield of phosphinate (**15**) (Scheme 5). In order to form the α amino phosphine oxide moiety, the best method available is to perform an Abramov–Pudovik like reaction through addition of metal phosphites[44–48] to aldehyde- and ketone-derived imines, or as many imines are unstable or difficult to purify, through the akin Lewis acid catalyzed one pot three-components reaction (starting from carbonyl compounds, amines and hydrogeno-phosphites).[49–53] The first step is thus to form the phosphinite (**16**) from phosphinate (**15**). Best results were obtained using Red-Al (aluminium bis(2-methoxyethoxy)sodium hydride).[54] ^1H and ^{31}P NMR analysis of the crude material showed that addition of 1.1 equiv hydride resulted in the complete conversion of (**15**) to (**16**). Yet, the modest 53% yield is related to the tedious purification of the phosphonite (Scheme 5).

Whichever the base, we used (*n*-BuLi, NaOEt, *i*-Pr_2Net or even P_4*t*-Bu phosphazene[9]), attempts to proceed to the deprotonation of phosphonite (**16**) were yet unsuccessful. On the other hand, trimethylsilylphosphinites are described to be very reactive species for Michaelis–Arbuzov reactions.[55] Indeed, protected α amino phos-



Scheme 4. Aldehyde (**7**) synthesis.

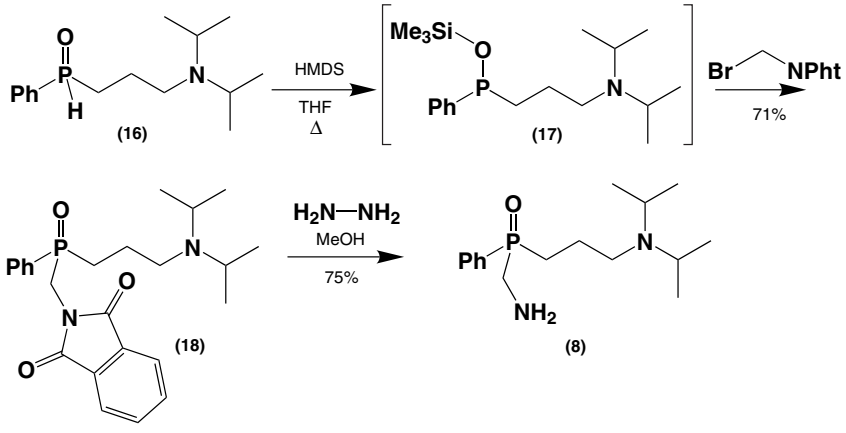


Scheme 5. Phosphonite (**16**) synthesis.

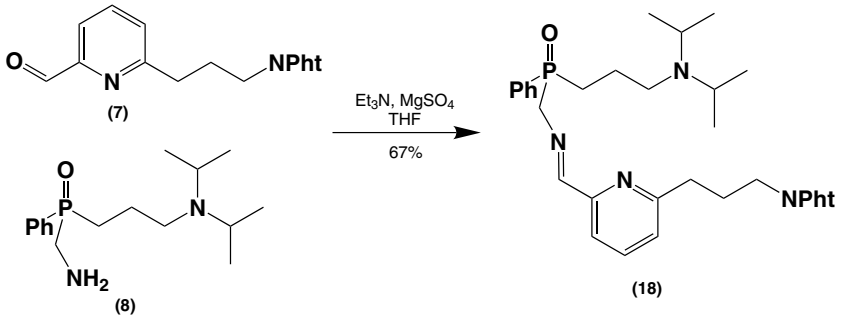
phine oxide (**18**) was formed in high yield when heating at reflux a THF solution of phosphinite (**16**) with hexamethyl disilazane and bromomethyl phthalimide through in situ formation of the trimethylsilylphosphinite (**17**).[56,57] Final deprotection of the phthalimide protective group afforded phosphine oxide (**8**), which was obtained in four steps and 22% yield from *O*,*O*-diethyl phenylphosphonite (Scheme 6).

Final coupling between aldehyde (**7**) and amine (**8**) was easily achieved mixing the two partners in THF under basic conditions (Et_3N), and with $MgSO_4$ acting as a water trap. Interestingly, imine (**18**) proved stable for days in a 1/1 THF–H_2O mixture and in phosphate buffer (pH 7.4), as followed by ^1H and ^{31}P NMR, yet any attempts to achieve the final deprotection of the *N*-phthalimide failed (Scheme 7).

Indeed, when (**18**) was submitted to 10 equiv *N*-butylamine in phosphate buffer (pH 7.4), imine slowly decomposed. Within 3 days, all of (**18**) had disappeared, showing that despite the stabilization through delocalization with the pyridine aromatic ring, imine moiety of hapten (**H1**) will be incompatible with



Scheme 6. Phosphine oxide (**8**) synthesis.



Scheme 7. Imine formation.

highly proteic media, and thus with the immunization process.

Current efforts are stressed upon the formation of a new hapten with the C–N double bond stabilized within an oxazole or thiazole ring. Synthesis and results of the immunization process will be reported in due time.

Acknowledgements

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