Detoxification of VX and Other V-Type Nerve Agents in Water at 37 °C and pH 7.4 by Substituted Sulfonatocalix[4]arenes

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Abstract: Sulfonatocalix[4]arenes with an appended hydroxamic acid residue can detoxify VX and related V-type neurotoxic organophosphonates with half-lives down to 3 min in aqueous buffer at 37°C and pH 7.4. The detoxification activity is attributed to the millimolar affinity of the calixarene moiety for the positively charged organophosphonates in combination with the correct arrangement of the hydroxamic acid group. The reaction involves phosphorylation of the hydroxamic acid followed by a Lossen rearrangement, thus rendering the mode of action stoichiometric rather than catalytic. Nevertheless, these calixarenes are currently the most efficient low-molecular-weight compounds for detoxifying persistent V-type nerve agents under mild conditions. They thus represent lead structures for novel antidotes that allow treatment of poisonings by these highly toxic chemicals.

With a percutaneous LD₅₀ value of 10 mg/human, the organophosphonate (OP) O-ethyl S-[2-(diisopropylamino)ethyl] methylphosphonothioate (VX; Figure 1) is one of the most toxic and most persistent chemical warfare agents.[3] Analogues that likewise have the amino group in the side chain and the relatively stable phosphono–thioester bond are similarly harmful.

The toxicity of nerve agents is mainly related to their high propensity to covalently modify a serine residue in the active site of the enzyme acetylcholinesterase (AChE).[2] In this form, AChE is unable to perform its function, namely to degrade the neurotransmitter acetylcholine, whose accumulation leads to severe toxic effects on the central and peripheral nervous system and ultimately to death.

A promising concept to treat poisonings by nerve agents involves the use of proteins, so-called bioscavengers, that detoxify OPs before clinical signs occur.[3] Bioscavengers have drawbacks, however, for example, low in vivo stability and immunogenicity, thus rendering synthetic scavengers attractive alternatives.[4] With only a few exceptions,[3] studies on synthetic scavengers have so far concentrated on cyclodextrin derivatives.[5] Their mode of action is expected to resemble that of proteins, with an initial complexation step that positions the phosphorus atom of the nerve agent close to a substituent on the cyclodextrin ring to facilitate the reaction. Despite notable success in this context,[6] cyclodextrins that detoxify V-type nerve agents have so far remained elusive. A possible explanation could be that V-type nerve agents are poor substrates for cyclodextrins because of their protonated side chain amino groups and, hence, polar nature at physiological pH values.[7] If this assumption is correct, hosts for ammonium ions in water should be more promising scaffolds for scavengers for V-type nerve agents. This idea is consistent with established design principles of supramolecular catalysts and reagents.[8]

Ajami and Rebek proposed the use of resorcinarene-derived cavitands as OP scavengers.[9] They described the synthesis of functionalized derivatives that could be used as a basis for such scavengers,[9] but detoxification studies were not reported. Our approach involved the use of sulfonatocalix[4]arenes that are also strong cation binders in water.[10,11] These calixarenes were decorated with a substituent containing a hydroxamic acid, which is known to detoxify OPs.[12,13] Synthesis involved the coupling of monoamine 1 to carboxylic acids with a protected hydroxamic acid. Deprotection followed by chromatographic purification and ion exchange afforded the products as sodium salts. Scheme 1 shows the synthesis of 2a as an example (for synthetic details, see the Supporting Information).

Of the compounds screened, 2a and 2b (Figure 2) exhibited highly promising properties. Analogues 2c–e were then prepared as reference compounds. In addition, the sulfamic acid derivatives 3a–c were considered to study the extent to which the calixarene moiety contributes to the activity of 2a and 2b.

![Figure 1](image-url). Structures of soman (GD) and the investigated V-type nerve agents.
Specifically, a nerve agent was incubated in Tris-HCl buffer (0.1 M, pH 7.40) at 37°C with an excess of a test compound. Aliquots of the solution were removed at regular time intervals to quantify their inhibitory effect on human AChE using a modified Ellman assay. AChE inhibition, described quantitatively by first-order inhibition rate constants $k_{inhib}$, decreased during incubation because of scavenger-mediated detoxification and spontaneous hydrolysis of the nerve agent. Fitting the time dependence of $k_{inhib}$ to monoeponential decay curves yielded rate constants $k_1$ that reflect how fast a nerve agent loses the ability to inhibit AChE (Figure 3). Correcting these rate constants for the rates of spontaneous hydrolysis, determined by performing the assay with the same OP in the absence of any scavenger, gave rate constants $k_{detox}$ which were used to calculate the half-lives $t_{1/2}$ of detoxification. Tris-HCl buffer was chosen as the medium because we typically also consider G-type nerve agents in the screening, whose spontaneous hydrolysis is too fast in phosphate buffer to allow reliable kinetic analyses. Selected measurements were also performed in phosphate buffer to assess the influence of the buffer on the detoxification of the V-type nerve agents. It is worth noting that all of our detoxification studies involved the use of real nerve agents and not of less toxic simulants.

Table 1 shows that the two calixarene-based hydroxamic acids 2a and 2b mediate VX detoxification with half-lives of about 5 min in Tris-HCl buffer, which is approximately 3500 times faster than the spontaneous hydrolysis of VX under the same conditions. VX detoxification induced by 2a is similarly fast in phosphate buffer. Analogues 2c and 2d, in which the hydroxamic acid group is methylated at the oxygen atom or replaced by a carboxylate group, respectively, are inactive. Shifting the hydroxamic acid group from the 3-position of the aromatic substituent in 2b to the 4-position in 2e also causes activity to disappear, and none of the reference compounds 3a–e lacking the calixarene unit detoxify VX within the timeframe of the measurement. Notably, the activity of compounds 2a and 2b is not restricted to VX; structurally similar VX metabolites like VX CVX VR VM could also be detoxified.

| VX   | CVX | VR | VM | n-propyl-VX | VE | i-propyl-VX | GD   |
|------|-----|----|----|-------------|----|-------------|------|
| 2a   | 3.7 ± 0.4 | 2.8 ± 0.1 | 3.4 ± 0.6 | 2.9 ± 0.5 | 2.4 ± 0.2 | 15.1 ± 0.4 | 17.0 ± 0.6 | 17.7 ± 0.9|
| 2a[b] | 2.9 ± 0.2 | 2.2 ± 0.1 | 2.3 ± 0.1 | n.d. | n.d. | n.d. | 5.8 ± 0.2 |
| 2b   | 7.5 ± 0.3 | 25.0 ± 0.7 | 28.6 ± 0.4 | 20.3 ± 0.4 | 25.3 ± 0.8 | 105.2 ± 4.7 | 40.5 ± 1.4 | 49.5 ± 3.4|
| 2c   | >120 | n.d. | n.d. | n.d. | n.d. | n.d. | >120 |
| 2d   | >120 | n.d. | n.d. | n.d. | n.d. | n.d. | >120 |
| 2e   | >120 | n.d. | n.d. | n.d. | n.d. | n.d. | 91.2 ± 6.8 |
| 3a   | >120 | n.d. | n.d. | n.d. | n.d. | n.d. | 27.2 ± 2.4 |
| 3b   | >120 | n.d. | n.d. | n.d. | n.d. | n.d. | 46.6 ± 2.8 |
| 3c   | >120 | >120 | >120 | >120 | >120 | >120 | 37.6 ± 4.1 |

[a] Determined in phosphate buffer (0.1 M, pH 7.4). [b] Not determined. [c] Detoxification too slow to allow determination of a reliable half-life.
related V-type nerve agents are also detoxified. The rates of
detoxification of the methyl phosphonates in the presence of
2a were almost equally fast, while the ethyl phosphonates VE
and i-propyl-VE are detoxified significantly slower. In the
case of 2b, the V-type nerve agents with linear alkyl groups in
the side chains are detoxified less efficiently than VX, and
going from the methyl phosphonates to the ethyl phospho-
nates causes another drop in activity. Equimolar mixtures of
the sulfanilic acid 3a and the calixarenes 1 or 2c proved to be
inactive, thus showing that the covalent linkage between the
calixarene unit and the substituent in 2a is required for
effective detoxification.

For comparison, the effects of the prepared compounds on
the detoxification of soman (GD) were also assessed. Table 1
shows that the O-methylated hydroxamic acid 2c also does
not mediate the detoxification of GD, which suggests that
the free hydroxamic OH group is required for the reaction. The
carboxylate group in 2d is presumably not sufficiently
nucleophilic to have an effect. All the other compounds
detoxify GD to some extent, irrespective of whether they
contain a calixarene moiety or not. The half-lives of GD
detoxifications by 2a and 3a or by 2b and 3b are very similar.
Thus, the detoxification of the neutral GD does not seem to
benefit from the calixarene ring. By contrast, this ring is
essential for the detoxification of the cationic V-type nerve
agents, as none of the sulfanilic acid derivatives exhibited
notable activity. The lack of activity of the simple hydroxamic
acids is consistent with the higher stability of phosphono-
thioates with respect to GD (the rate constants of sponta-
neous hydrolysis are $6.3 \times 10^{-3}$ min$^{-1}$ for GD and $5.2 \times
10^{-3}$ min$^{-1}$ for VX under the chosen conditions). The ability
of 2a and 2b to overcompensate this stability difference and
allow them to detoxify V-type nerve agents even faster than
GD, could, therefore, be an indication that the predicted
interactions between the calixarenes and cationic OPs indeed
facilitate detoxification.

To test this assumption, binding studies were performed
by using VX and the inactive calixarene derivative 2c as
binding partners. Qualitative information about complex
formation was obtained by comparing the $^1$H NMR spectrum of
VX with that of VX in the presence of 2 equiv of 2c in
phosphate buffer (0.1 M in D$_2$O, pH 7.81). Phosphate buffer
was used to avoid signals from organic buffer molecules in the
$^1$H NMR spectra. Figure 4 shows that the presence of 2c has
a pronounced effect on the resonances of the VX protons (for
the effect of complexation on the receptor signals, see
Figure S4). All the VX protons are shielded, with the
strongest upfield shift of 1.3 ppm observed for the signals of
the isopropyl-CH$_3$ groups. The shifts become smaller as the
distance of the respective proton from the diisopropylamino
group increases, but effects are even evident for the reso-
nances of the ethyl-CH$_2$ and P-CH$_3$ protons. Another notable
feature is the splitting of the isopropyl-CH$_3$ signal into two
doublets, which indicates that the stabilization of the tetrahe-
dral ammonium group of VX upon complexation causes the
isopropyl-CH$_3$ groups to become diastereotopic.

The profound signal shifts observed in the $^1$H NMR
spectrum are clear indications for the binding of VX to 2c.
Moreover, the extents of the shifts suggest that complex
geometries are preferred, with the cationic ammonium group
located inside the calixarene cavity. As a consequence, the
phosphorus atom of the nerve agent should be preferentially
oriented near the hydroxamic acid group of the scavenger,
thus facilitating reaction. An NMR titration under the same
conditions yielded a binding constant log $K_a$ of 4.11 ± 0.12 for
the complex between 2c and VX, thus confirming the
appreciable affinity of the sulfonatocalix[4]arene for posi-
tively charged nerve agents in water.

Efficient complexation of the nerve agents could, there-
fore, explain the detoxification activities of the calixarene-
based scavengers. The different activities observed for 2a, 2b,
and 2e may be related to differences in the nucleophilicities
of their substituents or to structural aspects of the complexes
formed. The former is less likely because benzohydroxamic
acid and corresponding pyridine derivatives do not typically
exhibit large differences in their $p$K$_a$ values or rates of
reaction in the presence of OP$_a$.[5b,12d] We therefore attribute
the higher activity of 2a with respect to 2b, and also the
different rates with which the investigated nerve agents are
detoxified, to structural effects of the respective complexes.
The loss of activity when moving the hydroxamic acid from the
3- to the 4-position of the aromatic substituent may
likewise be caused by the inability of the hydroxamic acid in
the VX complex with 2c to efficiently reach the phosphorous
atom of the nerve agent.

Information about the pathways underlying the detoxifi-
cation was obtained by following the reactions between VX
and calixarenes 2a and 2b by $^3$P NMR spectroscopy and
mass spectrometry. $^3$P NMR spectroscopy showed that the
spontaneous hydrolysis of VX (2.93 mm) in Tris-HCl buffer
(0.1 M, pH 7.40) at 37°C is associated with a progressive
decrease in the VX signal. Concomitantly, signals appeared
showing the formation of ethyl methylphosphonic acid (EMPA)
and the toxic metabolite of VX, S-(2-(diisopropyl-
 amino)ethyl) methylphosphonothioate (EA-2192). About
25% of the VX was hydrolyzed after 24 h under these
conditions, with the EMMA/EA-2192 ratio amounting to 5.7:1.

Incubating VX (2.93 mm) in Tris-HCl buffer (0.1 M,
pH 7.40) at 37°C with 1 equiv of 2a led to a notable 78% drop
in VX concentration within the first hour of the experiment
and a drop of 94% when using 2 equiv of 2a, thus
confirming the high detoxification ability of this scav-
The decrease in the VX concentration after 1 h was also significant in the case of 2b, but somewhat smaller. VX degradation was complete after 24 h in the presence of 2 equiv of either calixarene. In contrast, the residual VX reached a plateau at 5% and 20% when only 1 equiv of 2a and 2b was present, respectively, which could be attributed to product inhibition, as the 2-(diisopropylamino)ethanethiol group released during VX detoxification can be expected to bind to the calixarene ring with a similar affinity as VX. Importantly, scavenger-induced VX degradation is associated only with the formation of EMPA, thus indicating that the calixarenes mediate the selective cleavage of the P–S bond of VX and efficiently suppress formation of the toxic metabolite EA-2192. Since analogous results were obtained for 3a and 3c, the selectivity of the reaction seems to be characteristic for hydroxamic acids, as also observed in other studies,[14a] and not related to the calixarene moiety.

Mass spectrometry showed that VX degradation mediated by 2a is associated with the formation of amine 4 and Tris adduct 5 shown in Scheme 2. Analogous products were observed when 2b was reacted with VX. As a consequence, both scavengers are consumed during the reaction, thereby rendering their mode of action stoichiometric. The products observed indicate that the reaction includes a Lossen rearrangement, as also observed for the reaction of other organophosphates with hydroxamic acids.[12] The isocyanates resulting from this rearrangement are either hydrolyzed or react with nucleophiles, in our experiments buffer molecules, to yield the corresponding adducts.

Concluding, this study introduces a very promising concept for the development of synthetic scavengers for highly stable and persistent V-type nerve agents. Calixarenes 2a and 2b mediate effective detoxification of VX and other V-type nerve agents in aqueous buffer at 37°C and pH 7.4 with so far unmatched activity for synthetic compounds. The observed high detoxification rates can be attributed to the combined presence of a recognition unit that binds to cationic nerve agents and a properly arranged nucleophilic group that mediates the selective cleavage of the P–S bond of the nerve agent. Relating the activities of 2a and 2b to those of bioscavengers, for example, on the basis of *k*<sub>cat</sub>/*K<sub>M</sub> values, is difficult because the calixarenes are stoichiometric scavengers, while bioscavengers for VX act catalytically. Data compiled in a recent review show, however, that half-lives of detoxification in the range of a few seconds are required for scavengers to be potentially useful in vivo.[16b] Thus, 2a and 2b cannot replace bioscavengers yet, but their activities might allow them to be used to supplement current nerve agent therapies by shortening the period of toxicologically relevant poison concentrations. In addition, they should allow decontamination of skin or sensitive equipment under much milder conditions than those normally associated with VX detoxification.[11] Clearly, these calixarenes also represent highly promising lead structures for synthetic scavengers, whose activities can likely be improved in the future by fine-tuning both the recognition unit and the reactive group.

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Keywords: calixarenes · chemical warfare agents · detoxification · hydroxamic acids · scavengers

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