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Tabun scavengers based on hydroxamic acid containing cyclodextrins†

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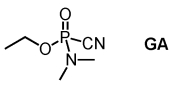
Arrangement of several hydroxamic acid-derived substituents along the cavity of a cyclodextrin ring leads to compounds that detoxify tabun in TRIS-HCl buffer at physiological pH and 37.0 °C with half-times as low as 3 min.

Tabun (ethyl dimethylphosphoramidocyanidate, **GA**) is a highly toxic organophosphate developed in 1936 and manufactured although never used as a chemical warfare agent during World War II. The toxic effects of **GA** are mainly related to the inhibition of acetylcholinesterase (AChE), the enzyme hydrolysing the neurotransmitter acetylcholine, by covalent modification of a serine residue in the protein's active site.[1] The mode of action of **GA** is thus closely related to that of other organophosphorus-derived nerve agents such as sarin, soman, or VX.[2]

Poisonings with organophosph(on)ates (OP) are typically treated by administration of atropine and certain oximes.[3] Atropine antagonises the action of acetylcholine at muscarinic receptors while the oximes reactivate inhibited AChE by cleaving the ester on the serine hydroxyl group. Because phosphonates formed with, for example, sarin or VX are more easily cleaved by oximes than the phosphoramidate formed by **GA**, therapeutic effects of oximes are smaller in the case of **GA** poisonings.[2]



GA

An alternative strategy to treat OP poisonings comprises degradation of the nerve agent prior to its reaction with AChE. In this context, scavengers on the basis of hydrolytically active enzymes have been developed[4] as well as those based on low molecular weight compounds.[5] Particular interesting lead structures for the development of low molecular weight scavengers are cyclodextrins because of their non-toxicity and well-known ability to mediate the hydrolysis of esters including those of phosphoric acid.[6–8] Indeed, β-cyclodextrin derivatives with oxime-derived substituents along the cavity have been shown to cause rapid detoxification of cyclosarin (within seconds under the conditions of the assay).[9] The activity is believed to benefit from inclusion of the cyclohexyl residue of the OP into the cyclodextrin cavity, which brings the cyclosarin phosphorous atom in close proximity to the appended nucleophilic group.

The effects of substituted cyclodextrin derivatives on **GA** degradation are usually smaller. A β-cyclodextrin containing one 3-carboxyl-4-iodosobenzyloxy residue in the 2-position of a glucose subunit detoxifies **GA** in TRIS-HCl buffer (pH 7.40) at 37 °C with a half-time of 8.4 min, for example.[8] Under similar conditions, a substituted β-cyclodextrin derivative containing an oxime group along the narrow opening has been shown by us to degrade **GA** with a half-time of 10.3 min.[10] With a *ca.* six-fold acceleration over the rate of spontaneous **GA** degradation the activity of this compound is far too low to render its use as a scavenger for the treatment of **GA** poisonings a realistic prospect.

Here we show that hydroxamic acids detoxify **GA** more efficiently than oximes. Although their ability to degrade OPs is known for some time,[11] hydroxamic acids have, to the best of our knowledge, not been used as nucleophilic groups in cyclodextrin-derived OP scavengers so far. According to our results these groups can lead to scavengers that detoxify **GA** at pH 7.40 and 37.0 °C with a half-time of *ca.* 3.0 min when appropriately arranged along the β-cyclodextrin cavity, which is a substantial improvement over previous systems.

While screening for cyclodextrin-based OP scavengers we came across β-cyclodextrin derivative **β^{6}1**, which exhibited a stronger effect on **GA** degradation than the previously prepared oximes.[10] With this new lead structure in hand we performed structural variations by arranging the hydroxamic acid residue on the secondary side of the β-cyclodextrin ring (**β^{2}1**, **β^{3}1**),‡ increasing the number of substituents (**β^{6}1$_2$**, **β^{6}1$_7$**), varying the cyclodextrin diameter (**α^{6}1$_6$**, **γ^{6}1$_8$**, see ESI† for structures), changing the structure of the hydroxamic acid (**β^{6}2**), or introducing methyl groups on one or both heteroatoms of the –NHOH group (**β^{6}3**, **β^{6}4**, **β^{6}5**, **β^{6}5$_2$**, **β^{6}5$_7$**). In addition, glucose derivatives **G^{6}1** and **G^{6}5** have also been synthesised to elucidate

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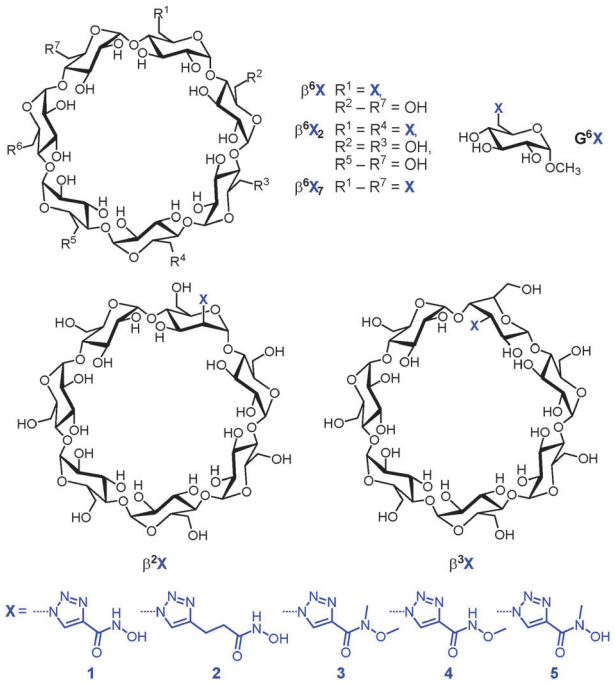


the effect of the cyclodextrin subunit on the degradation rate. All compounds were prepared from known azides and respective terminal alkynes by using the copper(I)-catalysed Huisgen cycloaddition and were isolated in analytically pure form (see ESI†).

The initial test to assess the effect on **GA** degradation involved incubation of the OP with an excess of a potential scavenger in TRIS-HCl buffer (pH 7.40) at 37.0 °C and estimation after 0, 30, and 60 min to what extent aliquots of these incubated samples inhibit AChE.[9,10,12] The inhibitory activity was related to the activity of a **GA** solution incubated in the absence of the test compounds. The resulting parameter Δk_1 correlates with scavenger activity: higher values for Δk_1 indicate higher activity. The results obtained are summarised in Fig. 1.

Fig. 1 shows that native β-cyclodextrin does not detoxify **GA** within the time frame of the assay. In contrast, all derivatives containing hydroxamic acid **1** are active, almost completely degrading the OP within 30 min. The position of the substituent on the cyclodextrin ring has obviously no large effect on degradation efficiency: compounds $\beta^6\mathbf{1}$, $\beta^2\mathbf{1}$, and $\beta^3\mathbf{1}$ are all similarly active.

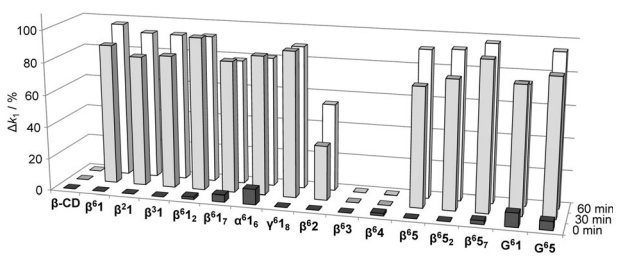


Fig. 1 Bar chart indicating the ability of the investigated hydroxamic acids to mediate **GA** degradation. Large columns indicate high activity. For reference the results obtained for native β-cyclodextrin (β-CD) are also shown. All results are means of $n = 3$ experiments. Standard deviation of a series of measurements with the same batch of AChE is <10%. Because the results can vary more strongly if different enzyme preparations are used we assume an overall error of 20%.

Not surprisingly, increasing the number of substituents on the β-cyclodextrin ring to 2 in $\beta^6\mathbf{1}_2$ and 7 in $\beta^6\mathbf{1}_7$ also yields active scavengers as does appending hydroxamic acid **1** to α- and γ-cyclodextrin as in $\alpha^6\mathbf{1}_6$ and $\gamma^6\mathbf{1}_8$. Compound $\beta^6\mathbf{2}$ decomposes **GA** only slowly indicating that the direct attachment of the hydroxamic acid group to the triazole moiety is crucial for activity. Methylation of the nitrogen and the oxygen atom of the –NHOH group ($\beta^6\mathbf{3}$) or of only the oxygen atom ($\beta^6\mathbf{4}$) yields completely inactive compounds clearly demonstrating that the OH group of the hydroxamic acid is involved in the degradation mechanism. Derivatives with a methyl group on the hydroxamic acid nitrogen atom ($\beta^6\mathbf{5}$, $\beta^6\mathbf{5}_2$, $\beta^6\mathbf{5}_7$) exhibit activity, however. Finally, it is important to point out that the glucose derivatives $G^6\mathbf{1}$ and $G^6\mathbf{5}$ are also rather active, suggesting that the cyclodextrin ring is not essential for **GA** degradation.

These results provided qualitative information about the activities of the whole set of investigated compounds showing that many of them possess promising properties. Exact rates of **GA** degradation were subsequently determined for compounds that exhibited restoration of more than 75% of AChE activity after 30 min of incubation in the qualitative assay. Quantitative measurements involved incubation of **GA** (1.0 μM in aqueous TRIS-HCl buffer at pH 7.40 and 37.0 °C) with a hydroxamic acid (500 μM) and determination of the inhibitory activity of this solution on AChE at defined intervals. After referencing the results to a control experiment, in which **GA** was incubated in the absence of the scavenger, pseudo first order rate constants k_{obs} were calculated from the resulting decay curves.[12] Rate constants and half times thus obtained are shown in Table 1.

Table 1 shows that all hydroxamic acids accelerate **GA** degradation significantly beyond the rate of spontaneous hydrolysis. With a half time of 8.6 min $\beta^6\mathbf{1}$ is almost 2 min more active than our best oxime-containing β-cyclodextrin.[10] Moving the hydroxamic acid to the secondary face of the ring causes a reduction in efficiency. Since there are no indications that the activity of $\beta^6\mathbf{1}$ is positively influenced by specific interactions between the cyclodextrin ring and the **GA** molecule because glucose derivative $G^6\mathbf{1}$ is practically as active as $\beta^6\mathbf{1}$, we attribute the lower activities of $\beta^2\mathbf{1}$ and $\beta^3\mathbf{1}$ to a more difficult accessibility of the hydroxamic acid group in these compounds.

Compound $\beta^6\mathbf{1}_2$ is more active than the monosubstituted analogue, consistent with the fact that the number of nucleophilic centres in solution increases by replacing $\beta^6\mathbf{1}$ with an

Table 1 Kinetic constants k_{obs} and half-times $t_{1/2}$ of **GA** degradation mediated by selected cyclodextrin and glucose derivatives[a]

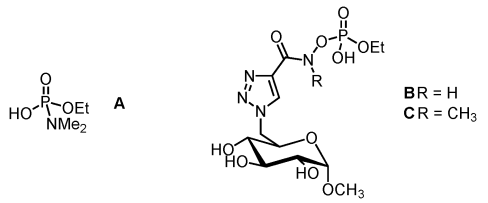
	$k_{obs} \times 10^2/\text{min}^{-1}$	$t_{1/2}$/min
Spontaneous hydrolysis	1.15 ± 0.04	60.3 ± 0.2
$\beta^6\mathbf{1}$	8.04 ± 0.11	8.6 ± 0.1
$\beta^2\mathbf{1}$	6.28 ± 0.14	11.0 ± 0.3
$\beta^3\mathbf{1}$	6.62 ± 0.30	10.5 ± 0.5
$\beta^6\mathbf{1}_2$	13.55 ± 0.33	5.1 ± 0.1
$\beta^6\mathbf{1}_7$	22.76 ± 1.08	3.0 ± 0.1
$\alpha^6\mathbf{1}_6$	17.88 ± 0.34	3.9 ± 0.1
$\gamma^6\mathbf{1}_8$	16.05 ± 1.06	4.3 ± 0.3
$\beta^6\mathbf{5}$	5.63 ± 0.04	12.3 ± 0.1
$\beta^6\mathbf{5}_7$	9.06 ± 0.29	7.6 ± 0.3
$G^6\mathbf{1}$	8.03 ± 0.13	8.6 ± 0.1
$G^6\mathbf{5}$	8.10 ± 0.25	8.6 ± 0.3

[a] In aqueous TRIS-HCl buffer at pH 7.40 and 37.0 °C, c(**GA**) = 1.0 μM, c(scavenger) = 500 μM.

equimolar amount of $\beta^6\mathbf{1}_2$. Raising the number of substituents on the ring even further causes an additional improvement in activity although the extent to which the activity of $\beta^6\mathbf{1}_7$ increases does not correlate linearly with the number of substituents. Since an approximate seven-fold increase in reaction rate was observed when the concentration of the glucose derivative $\mathbf{G^61}$ was increased from 500 μM to 3500 μM in the assay (see ESI†) it seems that not all of the seven substituents in $\beta^6\mathbf{1}_7$ are able to participate in **GA** degradation. Interestingly, the α- and γ-cyclodextrin derivatives $\alpha^6\mathbf{1}_6$ and $\gamma^6\mathbf{1}_8$ are somewhat less active than $\beta^6\mathbf{1}_7$ although the number of nucleophilic centres is even higher in the case of $\gamma^6\mathbf{1}_8$. The substituents are therefore presumably better arranged for reaction with **GA** in $\beta^6\mathbf{1}_7$ than in the larger or smaller cyclodextrin analogues. While the *N*-methylated glucose derivative $\mathbf{G^65}$ is as active as $\mathbf{G^61}$, the corresponding cyclodextrin derivatives $\beta^6\mathbf{5}$ and $\beta^6\mathbf{5}_7$ are less active than the non-methylated analogues.

GA degradation mediated by $\beta^6\mathbf{1}_7$ was additionally followed by using a GC-MS assay, which provided information about the enantioselectivity of the reaction.[13] This experiment yielded further evidence that interactions between the cyclodextrin ring and **GA** play no large role in the mode of action of $\beta^6\mathbf{1}_7$ because the two enantiomers of **GA** decompose with practically the same rate as observed for oxime-containing cyclodextrins.[10] The half time of 2.0 ± 0.1 min obtained in the GC-MS assay for the $\beta^6\mathbf{1}_7$-mediated **GA** degradation is in reasonable agreement with the result of the enzymatic assay.

Repeated treatment of $\beta^6\mathbf{1}$ or $\beta^6\mathbf{5}$ with an excess of **GA** revealed that both hydroxamic acids lose activity after the first round of reaction.[10] To obtain information about the nature of the products formed we followed **GA** degradation in TRIS-HCl buffer in the absence and presence of $\mathbf{G^61}$ and $\mathbf{G^65}$ using ^{31}P NMR spectroscopy and mass spectrometry. These measurements showed that spontaneous hydrolysis of **GA** in TRIS-HCl buffer practically exclusively yields hydroxytabun **A**. When 2 equiv. of $\mathbf{G^61}$ are present the OP not only disappears significantly more rapidly, no unreacted **GA** could be detected in the first NMR spectrum recorded *ca.* 30 min after sample preparation, but also the outcome of the reaction is different. Under these conditions, formation of a single product was observed, which represents phosphate diester **B** according to mass spectrometry lacking the CN *and* the NMe_2 group of **GA**. This product is expected to be relatively resistant towards further hydrolysis because of its anionic nature at physiological pH. Thus, reaction between $\mathbf{G^61}$ and **GA** results in irreversible covalent modification of the hydroxamic acid explaining why one scavenger molecule can induce degradation of only a single molecule of **GA**.



The course of the reaction between $\mathbf{G^65}$ and **GA** is somewhat more complex as it involves several transitional species (see ESI†). Although not all aspects of the underlying transformations have been fully elucidated yet our results indicate that diester **C** with a structure analogous to **B** is most likely the stable end product in this reaction.

In conclusion, we have shown that appending hydroxamic acid-derived substituents to cyclodextrin or glucose units affords potent scavengers for **GA**. Cyclodextrins with one hydroxamic acid residue do not possess higher activity than corresponding glucose derivatives, presumably because the cyclodextrin ring does not cooperatively contribute to the mode of action. Cyclodextrins represent, however, valuable scaffolds allowing introduction of several hydroxamic acid units. This strategy allows improvement of degradation efficiency while keeping the scavenger concentration constant. The results also indicate that our hydroxamic acids are irreversibly modified when reacting with **GA** with the course of the reaction depending on the actual nature of the acid. Work to elucidate the underlying mechanisms is ongoing to obtain information on how to improve the activity of these promising scavengers even further.

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Notes and references

‡ Note that the substituent is located in a mannose unit in $\beta^2\mathbf{1}$ and in an altrose unit in $\beta^3\mathbf{1}$. The presence of subunits in these compounds differing from glucose is due to the synthetic approach used for preparation.

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