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HIV-1 protease inhibition potential of functionalized polyoxometalates

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ABSTRACT

Polyoxometalates (POMs) are interesting biomedical agents due to their versatile anticancer and antiviral properties, such as remarkable anti-HIV activity. Although POMs are tunable and easily accessible inorganic drug prototypes in principle, their full potential can only be tapped by enhancing their biocompatibility, for example, through organic functionalization. We have therefore investigated the HIV-1 protease inhibition potential of functionalized Keggin- and Dawson-type POMs with organic side chains. Their inhibitory performance was furthermore compared to other POM types, and the buffer dependence of the results is discussed. In addition, chemical shift mapping NMR experiments were performed to exclude POM–substrate interactions. Whereas the introduction of organic side chains into POMs is a promising approach in principle, the influence of secondary effects on the reaction system also merits detailed investigation.

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Polyoxometalates (POMs) are transition metal oxide clusters that are preferably formed with W, Mo and V in their high oxidation states.[1] Their ever growing number of complex structural architectures brings forward an impressive application potential that attracts widespread research attention, for example, with respect to key technological applications,[2] (electro)catalytic processes,[3] magnetic materials[4] and nanotechnology.[5] Another special feature of POMs is their versatile bioactivity that leads to antibacterial, anticancer and antiviral properties.[6] These have been widely reviewed over the past years and POMs have been proven to be active against a wide range of viruses, including different influenza strains, Dengue fever virus and SARS coronavirus.[7] They have furthermore been tested as promising anti-HIV agents since the full outbreak of the AIDS epidemic in the 1980s. The first breakthrough was reported in 1988 when the first in vivo studies on humans were performed with the $[NaSb_9W_{21}O_{86}]^{18-}$ polyanion (commonly abbreviated as HPA-23).[8] However, HPA-23 caused severe side effects at higher dosages that forced the patients to discontinue the therapy.[8] Since then, the search for 'second generation' anti-HIV POMs with reduced toxicity was continued and the $[PTi_2W_{10}O_{40}]^{7-}$ polyanion (abbreviated as PM-19) was, for example, identified as a potential antiviral drug capable of inhibiting the replication of HIV-1[9] and herpes simplex virus (HSV)[10] in vitro.

Despite the multitude of empirical investigations on the antiviral activity of POMs, little is known about their interaction mechanisms with viruses or cells.[11] Concerning the anti-HIV activity, Inoue et al. investigated the inhibition of HIV-1 reverse transcriptase (RT) by various POMs.[12] Interestingly, cell culture experiments showed that the anti-HIV-1 activity of the POMs under consideration was not correlated with their HIV-1 RT inhibition.[12] A follow-up study by the same group identified the Keggin- and Dawson-POM types as possible 'lead compounds' for anti-HIV activity.[13] In 2001, a novel inhibition mechanism of HIV-1 protease by Dawson-type POMs was proposed in a pioneering study by Hill et al.[14] The high activity of the niobium-substituted tungstate α_2-$[P_2W_{17}NbO_{62}]^{7-}$ was explained with its binding to the flexible hinge regions on the surface of the HIV-1 protease. As most organic HIV-1 protease inhibitors bind into the active site, larger inorganic cluster molecules, such as POMs, would thus open up new ways of inhibition. Such alternative interaction pathways of POMs with HIV-1 protease would render them less sensitive towards the frequently occurring mutations of HIV-1, thereby circumventing the problem of therapeutic resistance in current AIDS therapy.[15] In addition, most anti-AIDS drugs are costly compounds that have to be administered in inconveniently high doses several times a day. POMs would offer economic advantage over the current anti-AIDS drugs in principle, because they can easily be synthesized from readily available precursors in a few synthetic steps.

The following years witnessed further reports on antiviral POMs[6a] and new insights were obtained regarding the interaction of POMs with human/bovine serum albumin[16] or protein kinase CK2.[17] Nevertheless, the fundamental issue of POM toxicity still remains to be overcome in order to turn them into powerful, versatile and low-cost inorganic anti-HIV agents. However, the development of hybrid and functionalized POMs is now proceeding rapidly[18] and thus provides new inspirations for research on inorganic anti-HIV agents together with the incorporation of antiviral

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POMs into biomacromolecular matrices as an additional strategy.[19] The increasing gap between these growing classes of novel POMs and their biomedical characterization inspired us to screen a selected variety of POM types with respect to their inhibitory effect on HIV-1 protease. Firstly, we compared the inhibitory potential of POMs with different cluster sizes. We then focused on Keggin- and Dawson-type POMs with different organic side chains in order to evaluate the influence of the functionalization on the inhibitory performance. In addition, we placed emphasis on methodological issues: the results are critically discussed with respect to their considerable buffer dependence. Furthermore, we present NMR studies in solution to exclude the possibility of POM–substrate interactions.

All POMs were synthesized according to published procedures and they are summarized in Table 1. Figure 1 illustrates the structural motifs (Keggin- and Dawson-type) for the majority of the investigated POMs and their organically substituted derivatives. They have emerged from recently developed novel functionalization approaches.[20] POM 1 has been included as a representative of 'sandwich-type' lacunary POM building blocks with embedded transition metal cations. POMs 2–5 are based on the recently discovered $\{Ln_6W_{63}\}$ cluster[21] as the largest polyoxotungstate type among the present selection. These compounds are of general interest, because the incorporation of lanthanoid cations into POMs is promising with respect to their potential double function as diagnostic (e.g., luminescence or MRI) and therapeutic agents.[16a,22] POM 20 (Preyssler type) bears resemblance to the Dawson type due to the arrangement of its five PW_5 units into a Dawson-related surface. POM 28 (Lindqvist type) as the most compact and smallest structure type rounds off the 'classic' POM spectrum.

The POMs were characterized by IR spectroscopy and other analytical methods if required (e.g., NMR or single crystal structure determination) and they were stable under the applied experimental conditions. HIV-1 protease was expressed in *Escherichia coli* BL21 (DE3) pLysS cells and purified by anionic exchange and size exclusion chromatography according to literature protocols (for further experimental details cf. Supplementary data).[23] Given that

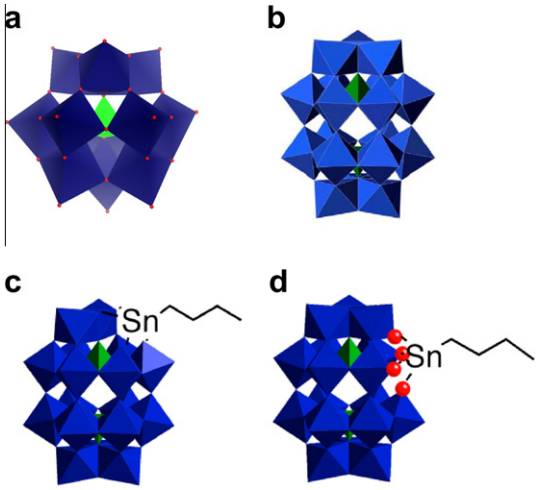

Figure 1. Overview of the most important POM types investigated in the present study: (a) Keggin type, (b) Dawson type, (c) α_2-substituted Dawson type, (d) α_1-substituted Dawson type.

HIV-1 protease has an autocatalytic activation site as well as autoprotolytic activity,[24] special attention must be paid to the high purity of the protein to precisely determine its concentration and to prevent possible interaction of the POMs with degraded protease fragments during the protease assays. For that reason, the substitution of one single amino acid, namely glutamine at position 7 to lysine (Q7K), has been performed to reach a 100-fold increase of the protein stability.[24] This residue is located in the first loop after the first β-strand in the protein and at a considerable spatial distance to the flexible hinge regions that were proposed as POM binding sites.[14] Therefore, it can be practically excluded that the use of the HIV-1 mutant Q7K affects the inhibitory potential of the POMs in comparison with the wild type. The introduction of the positively charged lysine might even exert a productive elec-

Table 1
Survey of the POMs screened for HIV-1 protease inhibition

No.	Formula	Cations	Type	Solvent	Ref.
1	$[Na_3Cu_3(H_2O)_9(AsW_9O_{33})_2]^{9-}$	Na_9	Dimer	H_2O	28
2	$[Cs\subset Tb_6As_6W_{63}O_{218}(H_2O)_{14}(OH)_4]^{25-}$	$Na_{21}Cs_4$	Hexamer	H_2O	21
3	$[Cs\subset Dy_6As_6W_{63}O_{218}(H_2O)_{14}(OH)_4]^{27-}\cdot Cl_2$	$Na_{22}Cs_5$	Hexamer	H_2O	21
4	$[Cs\subset Ho_6As_6W_{63}O_{218}(H_2O)_{14}(OH)_4]^{27-}\cdot Cl_2$	$Na_{22}Cs_5$	Hexamer	H_2O	21
5	$[Cs\subset Er_6As_6W_{63}O_{218}(H_2O)_{14}(OH)_4]^{25-}$	$Na_{21}Cs_4$	Hexamer	H_2O	21
6	α_2-$[P_2W_{17}O_{61}SnR]^{7-}$ (R = $C_3H_5O_2$)	TBA_6H	Dawson	DMSO	20
7	α_2-$[P_2W_{17}O_{61}SnR]^{6-}$ (R = C_4H_6O)	TBA_6	Dawson	DMSO	20
8	α_2-$P_2W_{17}O_{61}SnR]^{7-}$ (R = C_6H_8NO)	TBA_7	Dawson	DMSO	20
9	α_2-$[P_2W_{17}O_{61}SnR]^{7-}$ (R = C_4H_9)	TBA_6H	Dawson	DMSO	20
10	α_2-$[P_2W_{17}O_{61}SnR]^{7-}$ (R = $C_{18}H_{32}N_3O_5S$)	TBA_7	Dawson	DMSO	20
11	α_2-$[PW_{11}O_{39}SnR]^{4-}$ (R = $C_3H_5O_2$)	TBA_4	Keggin	DMSO	20
12	α_2-$[PW_{11}O_{39}SnR]^{4-}$ (R = C_4H_9)	TBA_4	Keggin	DMSO	20
13	α_2- $[PW_{11}O_{39}SnR]^{4-}$ (R = $C_{18}H_{32}N_3O_5S$)	TBA_4	Keggin	DMSO	20
14	α_1-$[P_2W_{17}O_{61}SnR]^{7-}$ (R = $C_3H_5O_2$)	TBA_6H	Dawson	DMSO	20
15	α_1-$[P_2W_{17}O_{61}SnR]^{7-}$ (R = C_6H_8NO)	TBA_7	Dawson	DMSO	20
16	α_1-$[P_2W_{17}O_{61}SnR]^{7-}$ (R = C_4H_9)	TBA_6H	Dawson	DMSO	20
17	α_2-$[P_2V_3W_{15}O_{63}R]^{7-}$ (R = $C_{13}H_{19}N$)	TBA_5	Dawson	DMSO	20
18	$[H_2W_{12}O_{42}]^{6-}$	$(NH_4)_6$	Individual	H_2O	23
19	$[NaSb_9W_{21}O_{86}]^{18-}$	$(NH_4)_{18}$	Individual	H_2O	29
20	$[NaP_5W_{30}O_{110}]^{14-}$	K_{14}	Preyssler	H_2O	29
21	α/β-$[P_2W_{18}O_{62}]^{6-}$	K_6	Dawson	H_2O	29
22	α_1-$[LiP_2W_{17}O_{61}]^{9-}$	K_9	Monolacunary Dawson	H_2O	29
23	α_2-$[P_2W_{17}O_{61}]^{10-}$	K_{10}	Monolacunary Dawson	H_2O	29
24	α_1-$[P_2W_{17}(NbO_2)O_{61}]^{7-}$	K_7	Dawson	H_2O	14
25	α_1-$[P_2W_{17}(NbO_2)O_{61}]^{7-}$	K_7	Dawson	H_2O	14
26	$[PW_{12}O_{40}]^{3-}$	NaH_2	Keggin	H_2O	30
27	$[PTi_2W_{10}O_{40}]^{7-}$	K_7	Keggin	H_2O	31
28	$[Pr(W_5O_{18})_2]^{9-}$	K_9	Lindqvist	H_2O	32

trostatic effect upon the binding of the highly negatively charged POMs to the protease. Standardized protease assays[25] were performed as described in the Supplementary data and an influence of POM absorbance on the measurements was excluded. The protease substrate was dissolved in dimethyl sulfoxide (DMSO) and two different buffer solutions were applied: buffer 1 contained a final DMSO concentration of 10%, whereas the DMSO content of buffer 2 was 1% (for details cf. Supplementary data). The stability of the protease during the assays was confirmed by Selwyn tests,[26] known as the standardized enzymatic stability test till date, under both buffer conditions. The inhibitory potential of all POMs was analyzed in triplicate measurements with Saquinavir as a standard inhibitor under both buffer conditions. Furthermore, the ingredients of the two different buffers were compared under the above conditions using POM 25 with a known high inhibitory activity[14] (cf. Table 1 and Table S-1). The higher concentration of DMSO and the additional detergent Nonidet P-40 obviously exerted the most significant influence on the measured inhibition (cf. Fig. S-1). Although the additional detergent Nonidet P-40 is supposed to stabilize the protease during the assay,[25] the enzymatic reaction also worked in the detergent-free buffer 2 with a lower DMSO concentration. Most importantly, these buffer additive tests show that the POM-induced protease inhibition is not a mere artefact arising from precipitation of protease or substrate during the reaction.

The relative activity of HIV-1 protease Q7K in buffers 1 and 2 is shown in Figure 2: the POMs were added in a 10- or 100-fold excess relative to the protease in buffer 1 and the relative protease activity remained higher than 70% in all reactions, whereas Saquinavir applied in an 1:1 ratio led to 100% inhibition. This excludes experimental setup errors and indicates that no inhibition of the enzymatic reaction by POMs occurred under these conditions. Especially for POMs 24 and 25, this result was unexpected in comparison with their previously reported high potential for HIV-1 protease inhibition.[14]

However, changing the conditions to buffer 2 completely altered the overall activity pattern. Although the use of detergent at 10% DMSO concentration had been reported to exert a stabilizing effect on HIV protease activity,[22] buffer 1 with 1% DMSO and less ingredients was sufficient to keep the protease active. A decline of the enzymatic reaction was observed in almost all experiments, in line with the preceding results:[14] POMs 21, 24 and 25 led to total protease inhibition as well as POMs 9, 12, 14 and 16. For a more detailed discussion of the results obtained in buffer 2, the POMs are classified into water-soluble POMs (1–5, 18–28) and functionalized DMSO-soluble POMs (6–17) with organic side chains. Interestingly, previous studies have pointed out that DMSO influences enzymatic reactions in a rather complex manner that varies widely—from stimulation to inhibitory effects—for the different systems under investigation.[27] However, this does not apply for the pristine HIV-1 protease in the present case and the DMSO dependence of POM-induced HIV-1 protease inhibition in contrast to its general inhibition by Saquinavir points to differences in the interaction mechanisms of organic and inorganic inhibitors, as had been suggested in earlier studies.[14]

A comparison of the water-soluble POMs with significant inhibitory potential (20–25) indicates that POMs related to the Dawson type are generally superior to the remaining POM architectures investigated in this study. POMs 21, 24 and 25 are Dawson structures and POMs 22 and 23 are monolacunary Dawson POMs (α_1- and α_2-isomers, respectively). In comparison with the Dawson type POMs, even the most prominent POMs, namely the aforementioned HPA-23 (POM 19) that was implemented in the first clinical studies[8] and the well-known anti-HIV agent PM-19 of the Keggin type (POM 27),[9] displayed a remarkably lower inhibitory effect.

Concerning POMs 2–5, their larger cluster size did not promote HIV-1 protease inhibition. Likewise, the dimeric structure of POMs 1 and 28 did not bring forward significant inhibitory activity—nor did the smaller $[H_2W_{12}O_{42}]^{6-}$ anion (POM 18) that performed even slightly worse than the Keggin compounds of related size (POMs 26 and 27). These results render the Dawson type especially promising for construction of functionalized antiviral POMs, provided that the above-mentioned buffer influence is taken into account. The dimensions of the Dawson cluster indeed appear to be in the appropriate range for POM/protein interactions as indicated in preceding theoretical studies.[14]

A more detailed comparison of the inhibitory effect among the DMSO-soluble POMs can be found in Figure 3: POMs 11–13 are Keggin type derivatives, whereas POMs 6–10 and 14–17 are Dawson type POMs that were functionalized with organic side chains at the α_1- (POMs 14–16) and α_2-position (POMs 6–10 and 17), respectively. A first glance at Figure 3 shows that the distinction between Keggin and Dawson type POMs is evened out through the introduction of organic residues: the Keggin type POM 12 with a butyl side chain induced complete inhibition of the protease activity (Fig. 3c). Generally, the introduction of a butyl side chain exerted the strongest inhibitory effect among the DMSO-soluble POMs, regardless of the POM framework or the Dawson regiosomer. This effect is even maintained with a 10-fold excess of the butyl-substituted POM 9. In the case of a propionic acid residue, the Keggin type derivative can as well compete with its Dawson analogs in terms of inhibitory effect (Fig. 3a). In parallel, the attach-

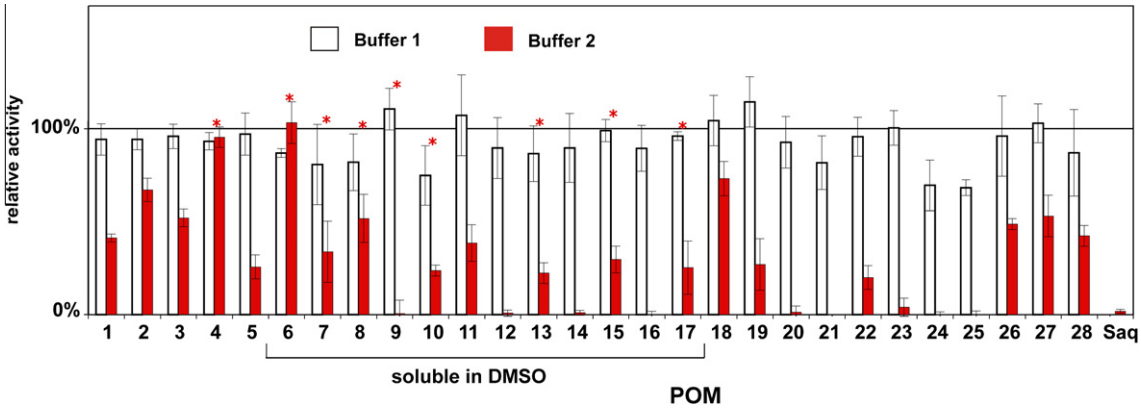

Figure 2. Relative activity of HIV-1 protease in the presence of different POMs as potential inhibitors (cf. Table 1). The reactions were performed in buffer 1 and buffer 2, respectively, and the total reaction volume was 200 μl. POMs were added to a concentration of 3 μM or 300 nM (asterisk). The activity of the protease in reactions without POMs was defined as 100% (Saq = Saquinavir).

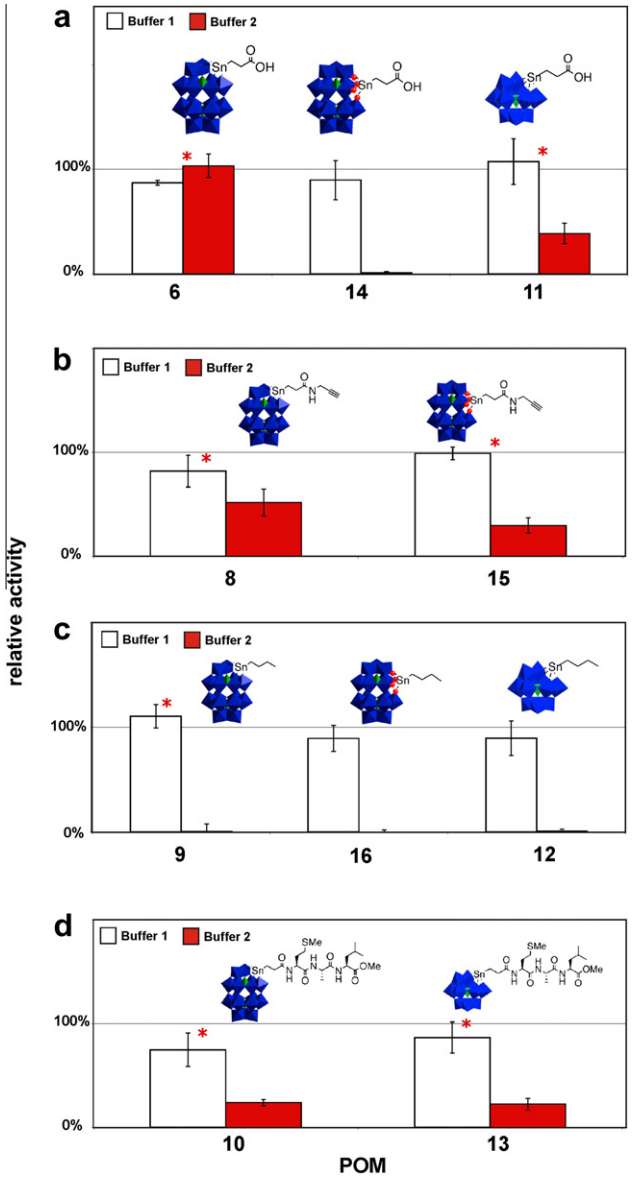

Figure 3. Relative activity of HIV-1 protease Q7K in reactions with DMSO-soluble POMs modified with organic side chains (red asterisks: POMs in 10-fold excess vs. protease, all other POMs were added in 100-fold excess); (a) POM 6, POM 14 and POM 11, (b) POM 8 and POM 15, (c) POM 9, POM 16 and POM 12, (d) POM 10 and POM 13.

ment of a more complex peptide-based side chain significantly reduced the protease activity irrespective of the POM host structure (Keggin or Dawson type, cf. Fig. 3d).

Although these results have to be interpreted with caution due to the limited number of POMs involved in the present study in comparison with the manifold experimental variables (such as buffer composition and POM concentration), their entirety indicates that the functionalization of POMs with organic moieties exerts a productive effect on their potential for HIV-1 protease inhibition.

All in all, the Dawson type POMs displayed the most promising overall inhibitory performance in the present study and it seems that the generally high antiviral potential of this 'classic' type is difficult to obtain with many newly discovered larger polyoxotungstates such as the investigated $\{Ln_6W_{63}\}$ type.[21] In this context, our recent luminescence studies on the interaction of $\{Eu_6W_{63}\}$ and $\{Tb_6W_{63}\}$ with human/bovine serum albumine[16a] also showed that their protein binding modes differ considerably from those of the smaller decatungstate cluster, thus pointing to the POM size as an important factor in structure–activity relationships. However, the introduction of organic side chains may be an important tool to modify structure activity relationships among the different POM cluster types in order to amplify the antiviral potential of POMs in general. This is supported by the fact that the introduction of a butyl group into the Keggin type (POM 12) drastically improves the inhibitory performance in comparison with the pristine Keggin POM 26. Such 'amphiphilic' POMs with unpolar organic substituents turned out to perform quite well in HIV-1 protease inhibition. For that reason, the inhibitory potential of the pristine organic compounds might also be tested as a reference.

However, this result also raises a critical point that should be taken into account for future studies: based on the present buffer additive tests, an interaction of the POMs with the substrate cannot be completely excluded. In order to detect possible interactions of the peptide substrate with POMs, we used solution NMR techniques. In particular, chemical shift mapping 600 MHz proton spectra were recorded on an approx. 100 μM peptide solution at pH 5.0 (100 mM acetate buffer) at 27 °C both in the absence and in the presence of POM 24 that displays significant HIV-1 protease inhibition.[14] The two samples were prepared from the same peptide stock solution, to one of which POM 24 was added. The pH was carefully controlled, and differed by less than 0.1 pH units in the two samples. The region of aliphatic protons displayed very little differences, whereas some amide protons experienced small shift changes. Nevertheless, the observed differences in the spectra were very minor (Fig. 4). From these changes we estimate that the K_d is likely to be in the molar range, strongly disfavoring a specific interaction with reasonable affinity and indicating the presence of a low-affinity, non-specific interaction. We conclude from these data that while a low-affinity non-specific interaction of the POM with the peptide does occur, its strength is so weak that it cannot be the reason for the observed inhibition of the substrate cleavage by the HIV-1 protease.

The ultimate proof of POM–protease interactions would certainly be the crystallization of a POM–protein complex. This challenging goal has never been reached over several decades of anti-HIV studies with POMs. Another option would be a pull-down experiment: POMs linked to beads that are incubated with HIV-1 protease or with substrate, respectively, followed by washing steps and analysis of the residues. This, however, would be a study in its own right, because POM/bead linking methods would have to be developed or, alternatively, HIV-1 protease would have to be stabilized during the coupling.

In summary, the present screening study outlines several methodological issues in the screening of POMs for anti-HIV-1 protease activity that have to be considered in follow-up experiments, namely the composition of the buffer system and the exclusion of POM/substrate interactions. Optimization studies, however, are now worthwhile, because we demonstrated that the functionalization of Dawson and Keggin type POMs with organic moieties is likely to enhance the inhibitory potential and to amplify the range of antiviral POM types, thus rendering them more biocompatible. Further studies concerning the cytotoxicity and cellular uptake of antiviral POMs are under way.

Acknowledgements

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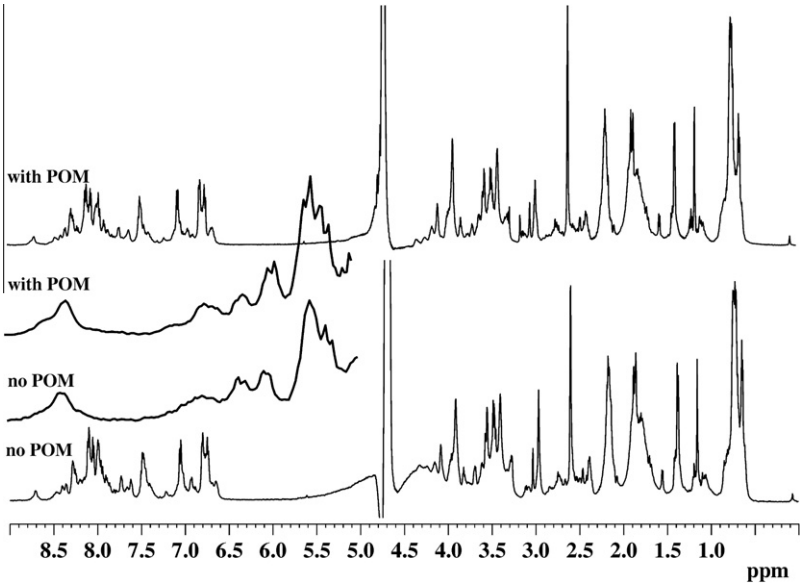

Figure 4. Proton spectra of the peptide substrate in absence (bottom spectrum) and presence (top spectrum) of POM 24, recorded at 600 MHz, T = 300 K. The expansion on the left displays the region from 8.2 to 8.8 ppm, and highlights the typically encountered minor changes in the spectra.

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Supplementary data

Supplementary data (expression and purification of HIV-1 protease, synthesis and inhibitor screening of POMs as well as additive screen test comparing the different buffers) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.12.103.

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