Effects of cucurbit[7]uril on enzymatic activity†

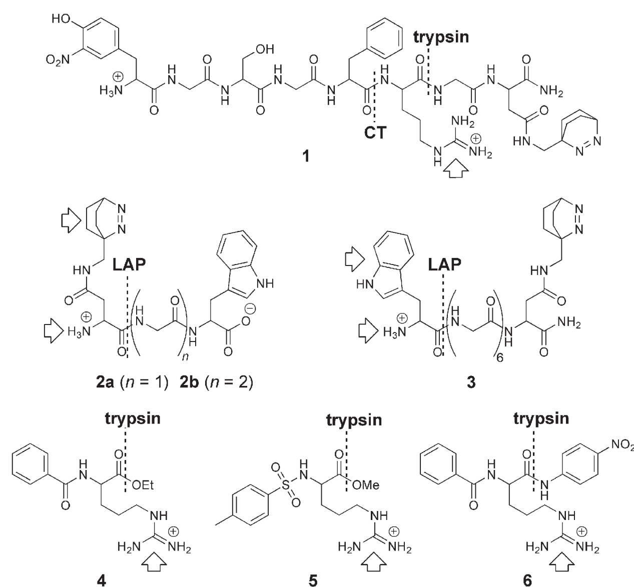
Andreas Hennig, Garima Ghale and Werner M. Nau*

Received (in Cambridge, UK) 22nd December 2006, Accepted 1st February 2007
First published as an Advance Article on the web 22nd February 2007
DOI: 10.1039/b618703j

The macrocyclic host cucurbit[7]uril exhibits highly specific inhibitory effects on the activity of proteases, which can be analyzed by a host–substrate complexation model.

Interactions of biomolecules (*e.g.*, peptides and proteins) with macrocyclic receptors are of current interest for sensor[1] and therapeutic applications[2–4] as well as enzyme-assisted synthesis.[5,6] While calixarenes[1,2] and cyclodextrins[3–5] have been frequently used in this context, studies with cucurbiturils are scarce,[7–11] although interesting differences between these classes of macrocyclic receptors have frequently been found. Herein, we compare the activity of a set of peptide model substrates (Scheme 1) towards the proteases chymotrypsin (CT), trypsin, and leucine aminopeptidase (LAP) in the absence and presence of cucurbit[7]uril (CB7). We demonstrate that the supramolecular complexation affords a highly efficient inhibition of several substrates, which has a direct bearing on drug delivery systems and potential biological effects.

Substrate hydrolysis was monitored by fluorescence (**1–3**) or absorbance (**4–6**) (see ESI†). In the absence of CB7, all proteases cleaved the model substrates with the expected rates (*cf*. Fig. 1 and Table 1). Addition of CB7 had an inhibitory effect on the activity of trypsin towards **1** and **6** and the activity of LAP towards **2–3**. In contrast, the activity of trypsin towards **4** and **5** as well as the activity of CT towards **1** was not significantly affected. The conservation of enzymatic activity for several protease/substrate combinations (*e.g.*, **4** and **5** with trypsin) and pronounced substrate-dependent variations (*e.g.*, **2** *vs.* **3** with LAP) revealed immediately that a complexation of the substrate and not an interaction with the enzyme was responsible for the inhibitory effect.



Scheme 1 Substrates for CT, trypsin, and LAP; dashed lines indicate cleavage sites, arrows indicate the presumed interaction sites with CB7.

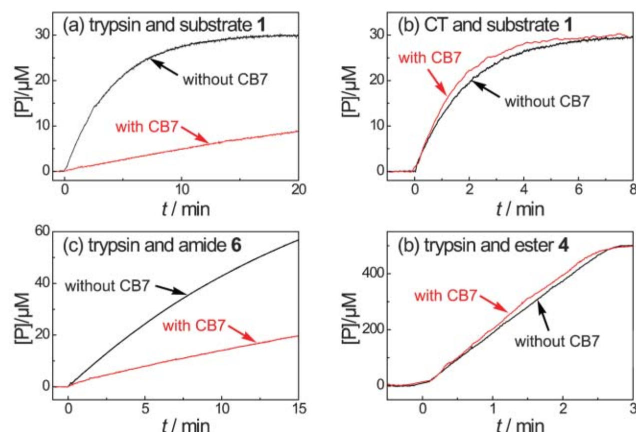


Fig. 1 Enzyme kinetic traces in the absence (black) and presence (red) of CB7 for the activity of (a) trypsin towards 30 μM peptide **1** (red: with 100 μM CB7), (b) CT towards 30 μM peptide **1** (red: with 1 mM CB7), (c) trypsin towards 500 μM amide **6** (red: with 1 mM CB7), and (d) trypsin towards 500 μM ester **4** (red: with 5 mM CB7).

Naturally, CB7 will bind to several sites of the large and polyfunctional substrates with different affinity. For example, control experiments by ^{1}H NMR, UV-Vis absorption, and fluorescence spectroscopy confirmed that also those substrates, whose activity was not influenced, were complexed by CB7 under the employed conditions. In addition, the exchange kinetics of CB7 is fast on the timescale of the enzymatic digestion.[10] We therefore introduced an *apparent* binding constant K_a (eqn (1)), assuming a 1 : 1 host–guest binding model for simplicity, as a measure of the complexation-induced inhibition. Accordingly (eqn (2)), the initial hydrolysis rate k_0 under equilibrium conditions is given by a linear combination of the cleavage rates for the uncomplexed (k_S) and complexed ($k_{S\cdot CB7}$) substrate, weighted by their molar fractions (x_S and $x_{S\cdot CB7}$). A plot of the initial rate *vs.* CB7 concentration provided K_a and $k_{S\cdot CB7}$ (Table 1).[12]

$$[S\cdot CB7] = \left([CB7]_0 - [S]_0 - 1/K_a\right)/2 + \sqrt{\frac{1}{4}\left([CB7]_0 - [S]_0 - 1/K_a\right)^2 - [CB7]_0[S]_0} \quad (1)$$

School of Engineering and Science, Jacobs University Bremen, Campus Ring 1, D-28759, Bremen, Germany. E-mail: w.nau@iu-bremen.de; Fax: +494212003229; Tel: +494212003233
† Electronic supplementary information (ESI) available: Details on assay procedures and NMR. See DOI: 10.1039/b618703j

 This journal is © The Royal Society of Chemistry 2007

Table 1 Effect of cucurbit[7]uril on the activity of proteases

Enzyme	Substrate	$k_S{}^a$/μM min^{-1}	$k_{S\cdot CB7}{}^b$/μM min^{-1}	K_a/10^3 M^{-1}
CT[c]	**1**	5.3	6.5[d]	<0.5
Trypsin[e]	**1**	6.0	<0.5	>50
LAP[f]	**2a**	1.8	<0.1	50 ± 20
	2b	5.3	<0.5	78 ± 35
	3	6.3	<0.9	2.8 ± 0.5
Trypsin[g]	**4**	210	240[h]	<0.5
	5	84	68[i]	<0.5
	6	5.5	<0.5	3.8 ± 0.6

[a] k_S was directly determined in the absence of CB7, error 10%. [b] $k_{S\cdot CB7}$ was fitted according to eqn (1) and (2) (10% error). [c] 10 μM **1**, 250 nM CT. [d] 1 mM CB7. [e] 30 μM **1**, 300 nM trypsin. [f] 10 μM **2–3**, 20 nM LAP for **2**, 150 nM LAP for **3**. [g] 500 μM **4**, 150 nM trypsin; 500 μM **5**, 30 nM trypsin; 100 μM **6**, 900 nM trypsin. [h] 5 mM CB7. [i] 3 mM CB7.

$$k_0 = x_S k_S + x_{S\cdot CB7} k_{S\cdot CB7} = k_S + (k_{S\cdot CB7}/[S]_0 - k_S/[S]_0)[S\cdot CB7] \quad (2)$$

Interestingly, in those cases where inhibition was observed, the fitted $k_{S\cdot CB7}$ values (the cleavage rates of the complexed substrates) became vanishingly small, such that only an upper limit is provided in Table 1. This suggests that complexation by CB7 leads to an efficient protection against enzymatic cleavage.

The inhibitory effect of CB7 on proteases needs to be considered in the context of its biological activity (*e.g.*, by suppression of proteolytic metabolic pathways) and for potential drug delivery applications.[8] For example, the inhibition by CB7 can be viewed as a stabilizing effect on the substrate against enzymatic degradation which could be of great interest for the delivery of peptide-based drugs.[3,4] Cyclodextrins, for example, are utilized as such drug stabilizing additives.[3,4] However, concentrations of up to 80 mM cyclodextrin, well above the presently used concentrations of CB7, are required to afford similarly stabilizing effects.[3b] In addition, the action of cyclodextrins is attributed to complexation of *hydrophobic* amino acid residues (Phe, Tyr, Trp) which serve as recognition sites for several hydrolytic enzymes. CB7, in its role as a cation receptor, is complementary because it has a high affinity for *positively charged* residues (Arg, Lys).

The latter conjecture was nicely confirmed by the contrasting effects of CB7 on substrate **1**, which contains well-known recognition sites for both, trypsin and CT. Trypsin recognizes the positively charged arginine, and its activity was efficiently suppressed at high CB7 concentrations (Fig. 1(a)), as would be expected from a complexation of the arginine residue. In contrast, cleavage of peptide **1** by CT, which specifically recognizes the hydrophobic phenyl residue of phenylalanine, was not inhibited (Fig. 1(b)), presumably because phenylalanine has only a very low affinity to CB7.[11]

In addition, cucurbiturils are known for their preferential complexation of positively charged *N*-terminal amino acids.[10,11] LAP cleaves off such *N*-terminal residues and we therefore trace the inhibition of substrates **2–3** back to a complexation of the *N*-terminal amino acid residues. Most likely, CB7 causes steric hindrance towards binding of the enzyme to the *N* terminus and "masks" (through ion–dipole interactions with the ureido carbonyl groups of the CB7 portal) the positively charged ammonium group, which are both critical for enzyme–substrate recognition.[13] The more than one order of magnitude difference between the K_a values for **2** and **3** (with exchanged terminal amino acids) supports this model. CB7 binds more strongly with the spherical 2,3-diazabicyclo[2.2.2]oct-2-ene residue (4×10^5 M^{-1})[7c] than with the Trp indole ring (2400 M^{-1} for Trp-OMe in Tris buffer at pH 7.8, this work), which results in an improved "protection" of the *N* terminus of substrates **2** towards cleavage by LAP compared to substrate **3**.

Surprisingly, the activity of trypsin towards the *esters* **4** and **5** was unaffected even with 3 mM CB7 (Fig. 1(b)), while hydrolysis of the *amides* **1** and **6** was inhibited as expected. Such a clear-cut differential reactivity of esters and amides has long been sought for when using proteases for organic synthesis, namely to increase the selectivity of their kinetically controlled hydrolysis reactions.[15] The observed selectivity of trypsin was the more surprising since NMR measurements (*cf.* ESI†) confirmed similar complexation patterns with CB7, *i.e.*, we observed strong upfield shifts of the aromatic protons, consistent with binding inside the cavity, and downfield shifts of the arginine side chain protons, suggesting portal binding.[14] The investigation of the underlying reasons for the contrasting inhibitory effects on ester and amide hydrolysis will consequently require the design of additional peptide model substrates in future studies.

In summary, cucurbiturils can inhibit the hydrolysis of substrates (and potentially drugs) towards LAP, trypsin, and other enzymes recognizing positively charged residues. This complements the use of cyclodextrins, which are used as stabilizers of drugs towards CT and other enzymes recognizing hydrophobic residues. Additionally, the observed effects of cucurbituril on protease activity are directly relevant for potential medicinal applications.

This work was performed within the graduate program "Nanomolecular Science". A. H. thanks Hoffmann-La Roche for a doctoral stipend.

Notes and references

1 R. Zadmard and T. Schrader, *J. Am. Chem. Soc.*, 2005, **127**, 904.
2 (*a*) Y. Hamuro, M. C. Calama, H. S. Park and A. D. Hamilton, *Angew. Chem., Int. Ed. Engl.*, 1997, **36**, 2680; (*b*) L. Memmi, A. Lazar, A. Brioude, V. Ball and A. W. Coleman, *Chem. Commun.*, 2001, 2474.
3 (*a*) W. J. Irwin, A. K. Dwivedi, P. A. Holbrook and M. J. Dey, *Pharm. Res.*, 1994, **11**, 1698; (*b*) K. Matsubara, Y. Ando, T. Irie and K. Uekama, *Pharm. Res.*, 1997, **14**, 1401; (*c*) J. B. Monteiro, L. D. Chiaradia, T. A. S. Brandão, J. Dal Magro and R. A. Yunes, *Int. J. Pharm.*, 2003, **267**, 93.
4 K. Uekama, F. Hirayama and T. Irie, *Chem. Rev.*, 1998, **98**, 2045.
5 (*a*) J. B. Harper, C. J. Easton and S. F. Lincoln, *Curr. Org. Chem.*, 2000, **4**, 429; (*b*) A. Koralewska, W. Augustyniak, A. Temeriusz and M. Kanska, *J. Inclusion Phenom. Macrocycl. Chem.*, 2004, **49**, 193.
6 J. Broos, R. Arends, G. B. van Dijk, W. Verboom, J. F. J. Engbersen and D. N. Reinhoudt, *J. Chem. Soc., Perkin Trans. 1*, 1996, 1415.
7 (*a*) J. Kim, I.-S. Jung, S.-Y. Kim, E. Lee, J.-K. Kang, S. Sakamoto, K. Yamaguchi and K. Kim, *J. Am. Chem. Soc.*, 2000, **122**, 540; (*b*) A. Day, A. P. Arnold, R. J. Blanch and B. Snushall, *J. Org. Chem.*, 2001, **66**, 8094; (*c*) C. Marquez, F. Huang and W. M. Nau, *IEEE Trans. Nanobiosci.*, 2004, **3**, 39.
8 (*a*) N. J. Wheate, A. I. Day, R. J. Blanch, A. P. Arnold, C. Cullinane and J. G. Collins, *Chem. Commun.*, 2004, 1424; (*b*) N. J. Wheate, D. P. Buck, A. I. Day and J. G. Collins, *Dalton Trans.*, 2006, 451.
9 H. Isobe, S. Sato, J. W. Lee, H.-J. Kim, K. Kim and E. Nakamura, *Chem. Commun.*, 2005, 1549.
10 M. E. Bush, N. D. Bouley and A. R. Urbach, *J. Am. Chem. Soc.*, 2005, **127**, 14511.

11 L. M. Heitmann, A. B. Taylor, P. J. Hart and A. R. Urbach, *J. Am. Chem. Soc.*, 2006, **128**, 12574.
12 M. McGarraghy and R. Darcy, *J. Inclusion Phenom. Macrocycl. Chem.*, 2000, **37**, 259.
13 W. T. Lowther and B. W. Matthews, *Chem. Rev.*, 2002, **102**, 4581.
14 W. L. Mock and N.-Y. Shih, *J. Org. Chem.*, 1986, **51**, 4440.
15 (*a*) S. Rival, J. Saulnier and J. Wallach, *Biocatal. Biotransform.*, 2000, **17**, 417; (*b*) F. Bordusa, *Chem. Rev.*, 2002, **102**, 4817.

Looking for that special chemical science research paper?

TRY this free news service:

Chemical Science

- highlights of newsworthy and significant advances in chemical science from across RSC journals
- free online access
- updated daily
- free access to the original research paper from every online article
- also available as a free print supplement in selected RSC journals.*

*A separately issued print subscription is also available.

Registered Charity Number: 207890

22030682

RSC Publishing

www.rsc.org/chemicalscience

This journal is © The Royal Society of Chemistry 2007