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Discovery of hydrolytic catalysts in a peptidocalixarene library by binding assay with a transition state analogue for the hydrolysis†

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Received (in Cambridge, UK) 13th July 2009, Accepted 5th October 2009
First published as an Advance Article on the web 15th October 2009
DOI: 10.1039/b913907a

Published on 15 October 2009. Downloaded by University of Rochester on 14/06/2013 20:41:09.

Hydrolytic catalysts were found in a peptidocalixarene library by binding assay with a transition state analogue for the hydrolysis. The rate of the reaction can be specifically enhanced up to 50-fold in the presence of the discovered catalyst.

The development of artificial enzymes that specifically catalyze the reaction with a target molecule is a challenging research area in the field of supramolecular chemistry.[1] Catalytic antibodies are established examples of tailor-made enzymes.[2] This approach has limited application, however, due to the instability of the antibodies under various reaction conditions (*i.e.*, solvent, temperature), because antibodies are high molecular-weight proteins. In addition, special equipment is required to prepare the catalytic antibodies. On the other hand, calixarenes are widely used as a platform for functional molecules, such as artificial receptors, sensors and catalysts.[3] Their potential function depends on the modification of the core calixarene. We previously synthesized calixarene-based peptide libraries to rapidly access desired functional molecules such as chemical sensors for specific oligopeptides.[4,5] We expected that catalysts for specific substrates would be easily found among the library in the same manner as the desired catalytic antibodies, because the library members can be postulated to function as antibodies.[6,7]

First, we chose hydrolysis of phenol ester **1** as a study model because it was the first successful system to generate a catalytic antibody with hydrolytic activity.[8] Phosphoric ester **4** was designed to mimic the tetrahedral geometry of the transition state for the hydrolysis of **1**. The aniline group in **4** was loaded as a reporter group to allow visualization at the time of the binding assay with previously synthesized resin-bound peptidocalixarene library **5**, which has 1000 members.[9] The transition state analogue **4** was synthesized from Fmoc-tyrosine in six steps (Fig. 1 and 2).

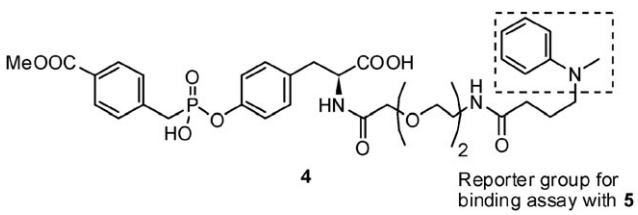
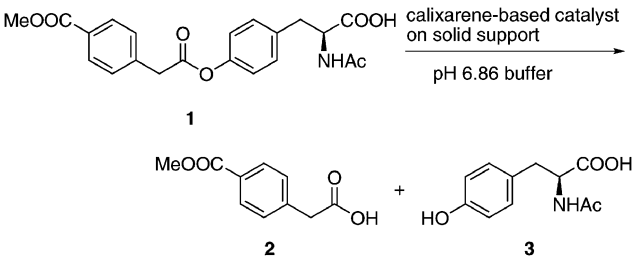


Fig. 1 Transition state analogue for the reaction shown in Scheme 1.



Scheme 1 Discovery of catalysts for the hydrolysis of **1**.

We incubated 3.5×10^{-3} mol L^{-1} of **4** with *ca.* 5 mg (*ca.* 3000 beads) of the library **5** in pH 6.86 phosphate buffer for 15 h to screen the library **5** for binding with **4**. The screening was performed by a post-labeling color assay utilizing the Trinder reaction.[10] Almost 5% of the beads were stained light purple as shown in Fig. 3. Thirty relatively dark colored beads were isolated and decoded to identify their amino acid sequences. The results are summarized in Table 1. All 30 beads had at least one basic amino acid residue (Arg, His, Lys). In particular, 20 of the 30 beads had His in AA_3. These findings suggested that, as expected, the binding nature in water is dominated by electrostatic interactions.

Four peptidocalixarenes **5a–d**, shown in Fig. 4, were selected and synthesized on beads in large amounts to estimate their hydrolytic activity. We mixed 1.25×10^{-3} mol L^{-1} of **1** with 0.2 equiv. peptidocalixarene **5a–d** in pH 6.86 phosphate buffer

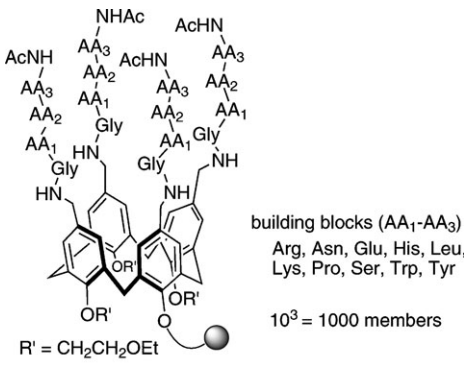


Fig. 2 Peptidocalixarene library **5**.

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† Electronic supplementary information (ESI) available: Experimental details for the synthesis of substrates **1**, **4**, **6**, **7**, **8** and **9**. Determination of kinetic parameters for the hydrolysis of **1**, **7** and **9**. See DOI: 10.1039/b913907a

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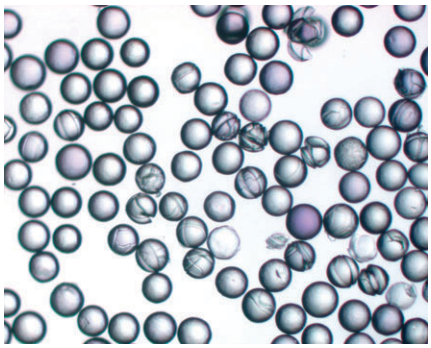
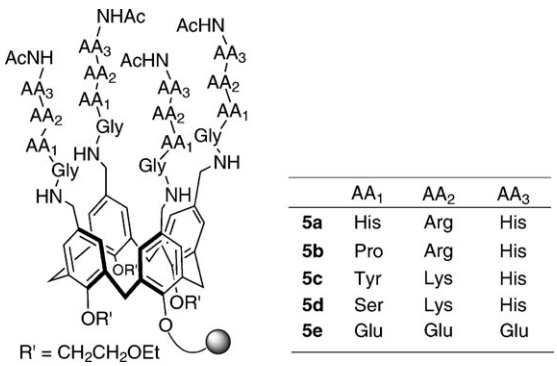


Fig. 3 Screening of the library **5** for binding with **4** (3.5×10^{-3} mol L^{-1}) by a post-labeling color assay utilizing the Trinder reaction.[9]

Table 1 Peptide sequences of the library members in **5** binding to a transition state analogue **4**[a]

Entry	AA_1	AA_2	AA_3	Frequency[b]
1	His	Arg	His	2
2	Pro	Arg	His	1
3	x_1[c]	Arg	His	4
4	Tyr	Lys	His	1
5	Ser	Lys	His	1
6	x_2[d]	Lys	His	4
7	x_3[e]	x_4[e]	His	7
8		Others		10
Total				30

[a] Exact sequences of all the 30 peptides are shown in ESI.† [b] Number of beads having the indicated sequences. [c] Any amino acid except His and Pro. [d] Any amino acid except Tyr and Ser. [e] x_3 and x_4 are combinations of any amino acids except for combinations appearing in entries 1–6.



	AA_1	AA_2	AA_3
5a	His	Arg	His
5b	Pro	Arg	His
5c	Tyr	Lys	His
5d	Ser	Lys	His
5e	Glu	Glu	Glu

Fig. 4 Selected candidates to catalyze the hydrolysis of **1**.

at 30 °C. The reaction was quantitatively monitored by HPLC. The concentration–time profile of the product **3** is shown in Fig. 5. The peptidocalixarenes clearly promoted the hydrolysis compared to the negative control **5e** or no beads. Despite their binding with transition state analogue **4**, their catalytic activities were significantly different from each other (Fig. 5); **5a** and **5b**, possessing Arg in AA_2, had a higher catalytic activity than **5c** and **5d** with Lys in AA_2. These results indicated that the combining site of the peptidocalixarenes binding with **4** is not necessarily the phosphoric ester function. The same phenomenon was reported in the case of catalytic antibodies coupled with a phage-display method.[11]

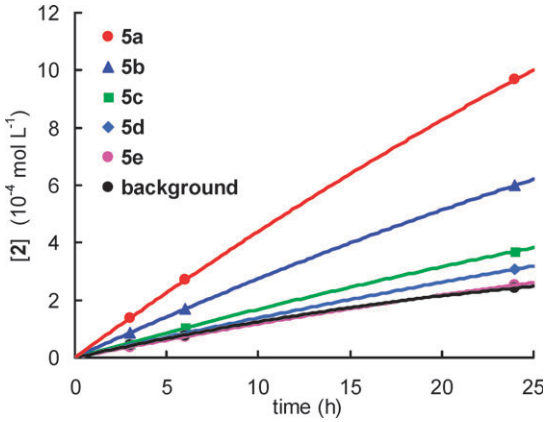


Fig. 5 Concentration–time profile of the hydrolysis of **1** (1.25×10^{-3} mol L^{-1}) with **5a–e** (0.2 equiv.). Solvent: aqueous phosphate buffer, pH 6.86, Temperature: 30 °C. The concentration of product **2** was determined by quantitative HPLC analysis.

Typical kinetic behavior based on the Michaelis–Menten principle was observed. The kinetic parameters, such as the Michaelis constant (K_m) and rate enhancement (k_{cat}/k_{uncat}), for the hydrolysis were calculated using a non-linear least-squares regression method (Table 2). An approximately 50-fold rate enhancement was observed in the presence of the peptidocalix[4]arene catalyst **5a**. If the transition state of the hydrolysis of **1** was stabilized by binding with **5a**, the reaction should be inhibited by the addition of the transition state analogue **6**. As expected, the reaction rate was clearly decreased. Lineweaver–Burk plots in the presence or absence of **6** are shown in Fig. 6. The two lines intersected in the left (– +) quadrant, indicating mixed-type inhibition, suggesting that the inhibitor **6** (Fig. 7) binds not only the active site for the hydrolysis, but also other sites of the peptidocalix[4]arene.

Table 2 Michaelis constant (K_m) and rate enhancement (k_{cat}/k_{uncat}) for the hydrolysis of **1**, **7** and **9** catalyzed by peptidocalix[4]arene **5a**

Substrate	K_m/mol L^{-1}	k_{cat}/k_{uncat}
1	1.59×10^{-3}	53.7
7	1.86×10^{-3}	42.8
9	4.02×10^{-3}	14.9

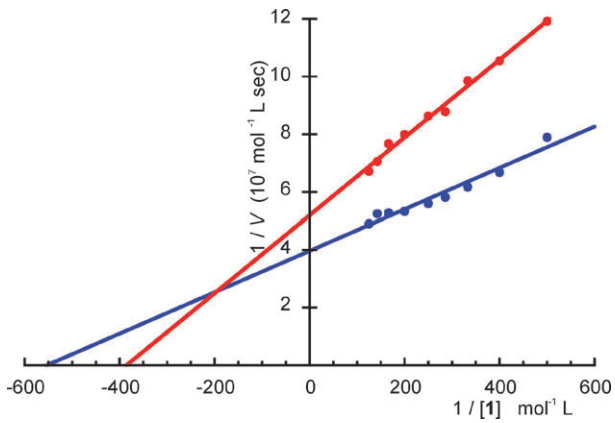
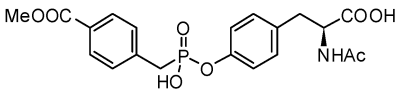


Fig. 6 Inhibition of hydrolysis of **1** by transition state analogue **6**. Lineweaver–Burk plots in the absence (blue) and the presence (red) of **6**.



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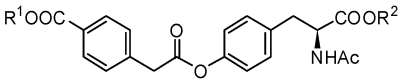

7: $R^1 = H$, $R^2 = Me$,
8: $R^1 = R^2 = Me$
9: $R^1 = R^2 = O(CH_2CH_2O)_2H$

Fig. 7 Structures of transition state analogue **6** without a reporter group and reference substrates **7**, **8** and **9** for hydrolysis catalyzed by **5a**.

This finding implies that all four peptide arms in **5a** are not involved in binding the substrate. The inhibition constant (K_i) was estimated to be 1.8×10^{-3} mol L^{-1}, which was calculated using a nonlinear least-squares regression method. The value was comparable to that of the Michaelis constant (K_m).

Finally, substrate specificity was investigated using reference compounds **7**, **8** and **9**. Compound **8** was poorly soluble under the same reaction conditions as those applied for **1**. K_m and k_{cat}/k_{uncat} for the hydrolysis of **7** and **9** are shown in Table 2. The K_m was slightly increased and rate enhancement was slightly decreased for monoester **7**. On the other hand, large differences in both values were observed for diester **9**, suggesting that catalyst **5a** discriminates between **1** and **7** or **9** and that the carboxylic acid in the substrates **1** and **7** is important for binding with **5a**.

In conclusion, hydrolytic catalysts for **1** were found in peptidocalixarene library **5** using a binding assay with transition state analogue **4** for the hydrolysis of **1**. The rate of the hydrolysis of **1** can be enhanced up to 50-fold in the presence of the discovered catalyst **5a**. This technique might be useful for creating tailor-made substrate specific catalysts. Design and synthesis of a large-sized peptidocalixarene library is in progress to find highly active and specific catalysts for various reactions.

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