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# COMBINATORIAL SEARCH OF SUBSTITUTED β-CYCLODEXTRINS FOR PHOSPHATASE-LIKE ACTIVITY

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**Abstract**: Thirteen per-6-alkylamino-6-deoxy-β-cyclodextrin libraries (β-CD libraries) were generated by a solution-phase combinatorial synthesis starting from per-6-iodo-6-deoxy-β-CD and different combinations of eleven individual amine nucleophiles. Certain libraries showed the ability to hydrolyze *p*-nitrophenyl phosphate in the presence of $Zn^{2+}$. 

Keywords: Enzymes and enzyme reactions; kinetics; cyclodextrins; combinatorial chemistry

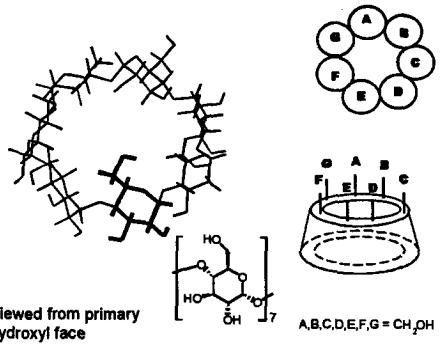


**Figure 1.** β-Cyclodextrin structure

Protein phosphorylation (kinase activity) plays an important biological role in many regulatory and signal transduction processes. It is also widely believed that uncontrolled signaling from protein tyrosine kinases can lead to diseases such as cancer, psoriasis, and atherosclerosis. Inhibitors that block the activity of protein tyrosine kinases are actively being sought as therapeutics for such disease states.[1] Alternatively, a small molecule capable of removing the phosphate group from tyrosine on such regulatory proteins (phosphatase activity) could represent a different approach to the treatment of these diseases. Several laboratories have studied molecules directed at the hydrolysis of phosphate esters,[2] including catalytic antibodies,[3] zinc azamacrocycles,[4] β-CD derivatives,[5] and combinatorially-based polyallylamide libraries.[6]

The shape of β-CD is reminiscent of a lampshade or truncated cone with a hydrophobic pocket at its center which can form inclusion complexes with various hydrophobic guest molecules (Figure 1). With appropriate modification of the primary hydroxyl face, this pocket has been shown to mimic the active sites of many enzymes.[7] Tabushi has sug-

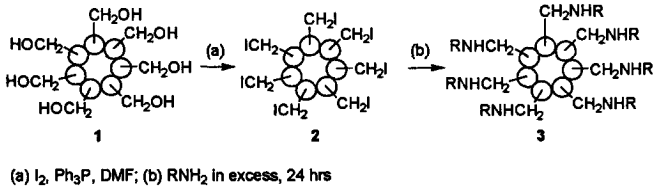


**Figure 2.** Library syntheses (See Figure 3 for amine reactants)

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gested that the key to developing these artificial enzymes is to "prepare a series of modified CDs, each of which has appropriate functional groups exactly at the required spatial environment (e.g. distance or angle between functional groups)".[7a] However the subilities of molecular recognition are difficult to predict *a priori*, and the chemistry for preparing regiospecific β-CD isomers is only in its infancy. Herein we report a combinatorial approach to the search for phosphatase-like activity from substituted β-CDs. Thirteen β-CD libraries, totaling approximately 28,000 compounds ($3^7$ x 13 = 28,431, see Table 1), were generated from β-CD (Figure 2) with eleven individual amines (Figure 3). Kinetic data for *p*-nitrophenyl phosphate (*p*-NPP) hydrolysis of each library are illustrated in Table 1.

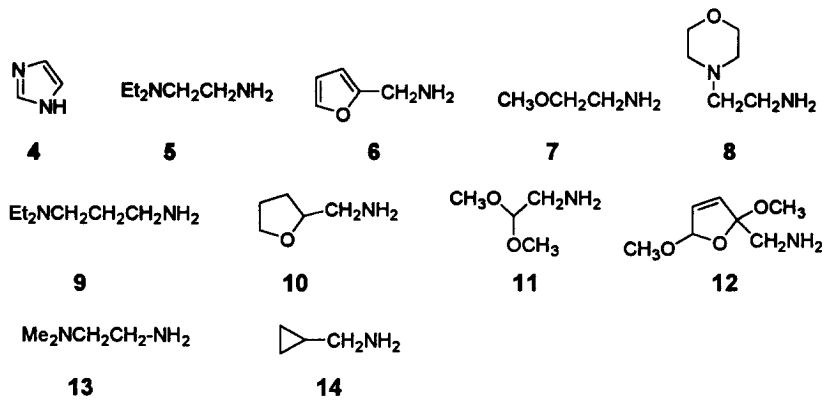


**Figure 3.** Amines used in library syntheses

Prior to library synthesis the least active nucleophile in these syntheses, imidazole (4), was used to op-

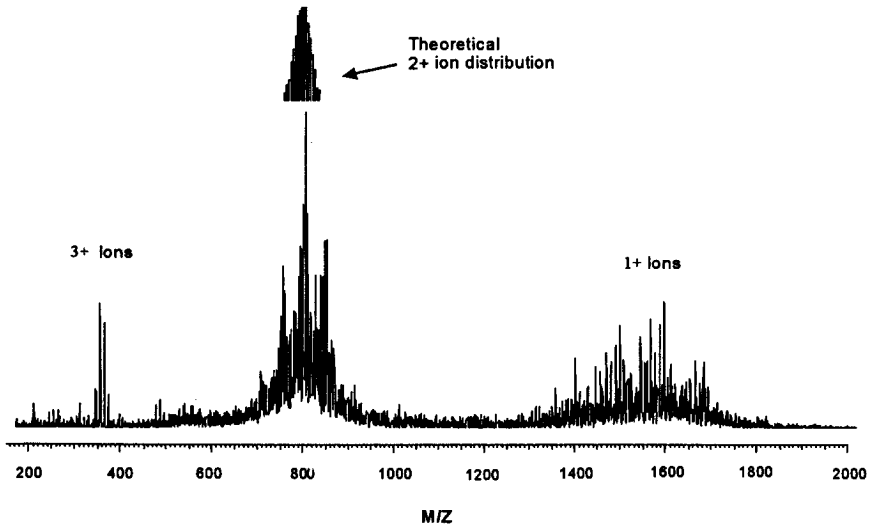


**Figure 4.** Electrospray mass spectrum of most active β-CD library **X**

timize reaction conditions and subsequent work-up procedures. Per-6-iodo-6-deoxy-β-CD (**2**) was prepared according to the literature[8] followed by nucleophilic displacement of iodine with imidazole (10 mole excess relative to each iodine) in DMF at 80 °C for 24 hours, similar to chemistry used by Thatcher *et al.*[9]

**Table 1.** Hydrolysis of *p*-NPP catalyzed by β-CD libraries in the presence of $Zn^{2+}$ at 37 °C [a]

| Library | Amine reactants[b] | $\bar{k}_{obs}$ ($s^{-1}$) [c] | $\bar{k}_{rel}$ [d] |
|---|---|---|---|
| Blank | None | $2.37 \times 10^{-7}$ | 1.0 |
| **I** | **4, 5, 6** | $(1.05, 1.06) \times 10^{-6}$ | 4.5 |
| **II** | **4, 7, 8** | $(5.19, 5.26) \times 10^{-7}$ | 2.2 |
| **III** | **4, 6, 9** | $(2.47, 2.42) \times 10^{-7}$ | 1.0 |
| **IV** | **4, 6, 8** | $(4.06, 4.37) \times 10^{-7}$ | 1.8 |
| **V** | **4, 5, 8** | $(8.46, 8.26) \times 10^{-7}$ | 3.6 |
| **VI** | **4, 8, 10** | $(3.26, 3.11) \times 10^{-7}$ | 1.4 |
| **VII** | **4, 5, 11** | $(1.68, 1.68) \times 10^{-6}$ | 7.2 |
| **VIII** | **4, 5, 10** | $(5.16, 4.95) \times 10^{-7}$ | 2.2 |
| **IX** | **7, 10, 12** | $(9.49, 9.18) \times 10^{-7}$ | 4.0 |
| **X** | **4, 6, 13** | $(1.58, 1.53) \times 10^{-5}$ | 66.7 |
| **XI** | **4, 10, 13** | $(7.29, 7.53) \times 10^{-6}$ | 31.7 |
| **XII** | **6, 13, 14** | $(3.22, 3.22) \times 10^{-6}$ | 13.8 |
| **XIII** | **4, 13, 14** | $(4.57, 4.75) \times 10^{-5}$ | 19.9 |
| Cyclodextrin [e] | None | $(4.16, 3.96) \times 10^{-7}$ | 1.7 |
| Side chains [f] | None | $(2.78, 2.95) \times 10^{-7}$ | 1.2 |
| Cyclodextrin + side chains [g] | None | $(6.22, 6.26) \times 10^{-7}$ | 2.7 |

[a]All solutions were 0.5 mg library in 1 mL $H_2O$ ($3 \times 10^{-4}$ M calculated from average molecular weight of library members), $2.40 \times 10^{-3}$ M $ZnCl_2$, and $1.73 \times 10^{-4}$ M substrate. Reactions were run in duplicate. [b]Amine structures are given in Figure 3. [c]Pseudo-first-order rate constants were calculated by the initial slope method. [d]Rate constants relative to blank (buffer + $Zn^{2+}$). [e]Cyclodextrin is $3 \times 10^{-4}$ M. [f]Side chains of **X** were used alone, each at $7 \times 10^{-4}$ M. [g]Cyclodextrin is $3 \times 10^{-4}$ M. Side chains of **X** were used, each at $7 \times 10^{-4}$ M.

After removal of most of the DMF under reduced pressure, crude per-6-imidazoyl-6-deoxy-β-CD was obtained by precipitating the residue with ethyl acetate, filtering, and washing. This product was sonicated in ethyl acetate for 10 min to give a fine powder, which was further stirred for an hour and filtered to give the final product (ESMS = 1485.7, 743.3, 496.1 m/z for the 1+, 2+, and 3+ [per-6-imidazoyl-6-deoxy-β-CD+nH]$^{n+}$ ions) after through drying in *vaccuo*. The yield of this product was judged to be 84% after conversion to the per HI salt. The latter was prepared by titrating the product with aqueous HI to pH 3 and then removing solvent and excess HI in *vaccuo*.

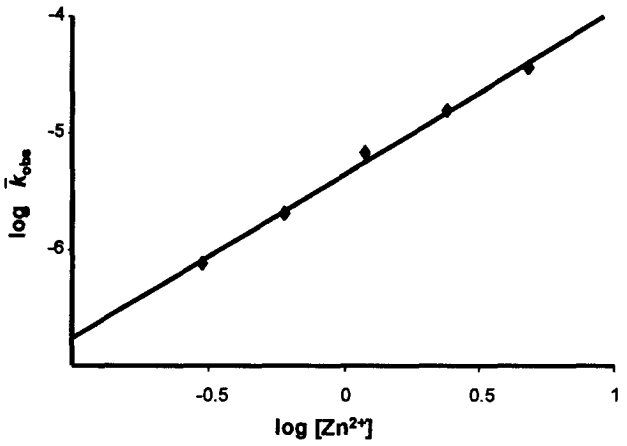


**Figure 5.** Log-log plot of $[Zn^{2+}]$ versus $\bar{k}_{obs}$ for *p*-NPP substrate hydrolysis by β-CD library **X**.

Library syntheses were achieved using these same model reaction conditions and purification procedures. Excess amines (each amine/reactive iodine = 10) were used in an attempt to get a statistical distribution of amines on the primary face of the β-CD scaffold. Thirteen β-CD libraries (**I-XIII**, Table 1) were prepared by selecting three amines at a time from the eleven amines given in Figure 3. Selected libraries were examined by ESMS against theoretically calculated spectra.[10] As shown in Figure 4 the doubly charged ion envelope of the most active library **X** (showing the greatest phosphatase-like activity) was similar to that calculated theoretically, the latter assumed a statistically equal distribution of each of the reactant side chains.

The thirteen β-CD libraries (**I-XIII**) were evaluated against controls for the ability to hydrolyze *p*-NPP in Tris buffer in the presence of $Zn^{2+}$ at 37 $^{0}$C.[11] Rate-enhancements ( $\bar{k}_{rel}$) relative to background (buffer + $Zn^{2+}$ = blank) by the initial slope method are given in Table 1 for the thirteen libraries and related controls. The bars over these classical constants denote the fact that these activities are an average of the activities of all active constituents in the library. The best library **X** catalyzed *p*-NPP hydrolysis with a rate constant of $4.2\times10^{-6}$ $s^{-1}$, which is about 7 times larger than $\bar{k}_{obs}$ reported for a combinatorially-based polyallylamide library,[6] and about 36 times larger than $k_{obs}$ reported for a catalytic antibody.[3] Controls with β-CD, the side chain amines alone, and a mixture of β-CD and side chain amines showed little if any phosphatase-like activity relative to the blank. Particularly noteworthy is the sixty-fold reduction in phosphatase activity seen when the methyl group (library **X**) is changed to an ethyl group in the common N,N-dialkylethylenediamine side chain (library **I**). Comparison of libraries **X** and **XI** indicates that saturation of the furfurylamine decreased hydrolytic activity by half, while replacement of imidazole (library **X**) with cyclopropylamine (library **XII**) decreased hydrolytic activity by 85%. Such "structure activity relationships" suggest underlying specificity of interaction of the *p*-NPP substrate with the substituted β-CDs responsible for the phosphatase-like activity.

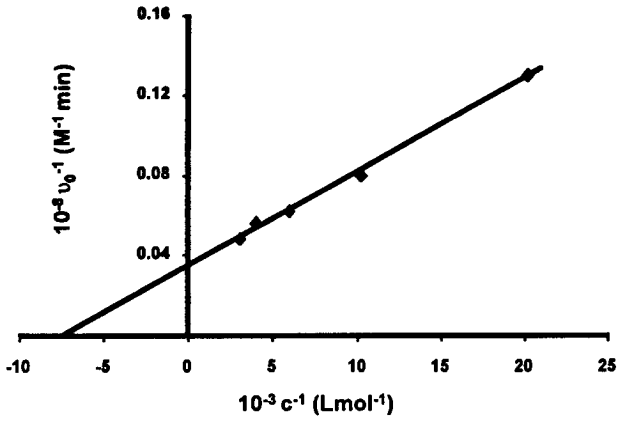


**Figure 6.** Double-reciprocal plot of β-CD library X with *p*-NPP as substrate.

In order to better define the role of $Zn^{2+}$ in the phosphatase activity, *p*-NPP hydrolysis was performed at five different concentrations of zinc. As shown in Figure 5, a plot of log $\bar{k}_{obs}$ versus log [$Zn^{2+}$] gave a straight line ($r^2$ = 0.996) with a slope of 1.4 from 4.8 to 0.3 mM $Zn^{2+}$. For each active member there is a specific number of $Zn^{2+}$ ions participating in the transition state (assuming one substituted β-CD per transition state). Consequently, the observed value of 1.4 $Zn^{2+}$ projects a transition state involving more than one mechanism, with at lease one mechanism including more than one $Zn^{2+}$.

As shown in Figure 6, hydrolysis of *p*-NPP by library X was also subjected to Michaelis-Menten analysis. The double-reciprocal Lineweaver-Burk plot gave a $\bar{V}_{max}$ of $2.85\times10^{-7}$ M min$^{-1}$ and a $\bar{K}_m$ of 134 μM ($r^2$ = 0.998). Thus the turnover number is from 2.1 min$^{-1}$ (only one active member in the library) to $9.5\times10^{-4}$ min$^{-1}$ (all library members are equally active). Although the turnover number of $9.5\times10^{-4}$ min$^{-1}$ is small, this value is comparable with that reported for the catalytic antibody.[3]

In summary, we have prepared thirteen β-CD libraries, estimated to contain about 28,000 compounds in total, by the use of solution-phase combinatorial chemistry techniques. Significant phosphatase-like activity was seen with several libraries, and seemingly small changes in side chain reactants produced significant changes in this activity. Hydrolysis of *p*-NPP with the best library reveals typical Michaelis-Menten kinetics.

Preliminary kinetic data have shown that tri-substituted β-CD libraries using the same strategy also have phosphatase-like activity. We are presently pursuing these libraries which hold promise for being deconvoluted to yield agents with specific phosphatase activity against phosporylated peptides.

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10. All mass spectra were taken on a Finnigan LCQ ion trap mass spectrometer (cap. = 40 V, cap. temp = 200 °C). For the per-imidazole derivative 8% collision energy was used. Samples (10 μg/mL in MeOH:water, 1:2) were infused at 5-10 μL/min.
11. 800 μl of Tris buffer (pH 7.4, 62.5 mM in $H_2O$) containing 3 mM $Zn^{2+}$ and 100 μL of *p*-NPP (1.73 mM in $H_2O$) were mixed well in the cuvette. Then cuvette was sealed with Teflon stopper and brought to 37 °C in the cell block of the absorbance spectrometer. After 15 min equilibration time, 100 μL of library (5 mg in 1 mL $H_2O$) was added into the cuvette and the solution was mixed thoroughly. The increase in concentration of nitrophenylate ($\lambda$= 400 nm) was measured every 3 min. In all cases, the reported rate constants were calculated by the initial slopes method (< 5% conversion, > 25 points) with extinction coefficient of nitrophenylate ($\varepsilon$= $1.68\times10^4$ $M^{-1}cm^{-1}$ at 400 nm), which was measured under identical conditions.