Report

Combinatorial de Novo Synthesis of Catalysts: How Much of a Hit-Structure Is Needed for Activity?

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Reports

Combinatorial de Novo Synthesis of Catalysts: How Much of a Hit-Structure Is Needed for Activity?

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Introduction

The development of new catalysts is of high importance for both academic and industrial chemistry. To render this time- and cost-intensive process more efficient, combinatorial methods have been introduced to the field within the past few years.[1−4] Here we describe the combinatorial discovery of a novel system for the cleavage of phosphoric diesters.[4,5] The overall procedure includes the split-mix synthesis[6−8] of a peptide-encoded library of 1458 solid-phase bound ligands, complexing of the ligand library with metal ions, and screening of the library with two newly developed assays for the detection of solid-phase bound cleavage activity. We chose the cleavage of phosphodiesters as the reaction of interest due to the importance of artificial phosphodiesterases for molecular biology and biotechnology.[4,5] Artificial phosphodiesterase activity was found in the presence of zinc and europium ions. A structure−activity analysis of two of the ligands revealed that the complete, polymer bound ligand is necessary for activity in the zinc/ligand system, whereas polymer bound histidine was found as the crucial substructure in combination with europium.

Results and Discussion

The general structure of the ligand library (containing a total of 1458 ligands) is outlined in Figure 1. In all three sublibraries, the linear ligand strand starts with a tripeptide zone (AS^1-AS^2-AS^3), composed of L-Arg, L-Lys, and L-His in a combinatorial manner. Arginine and lysine were chosen because they activate phosphates by electrophilic catalysis in a number of enzymes.[5,9] Histidine is well known as an acid−base catalyst and as a ligand in many metallo enzymes.[5,9,10] This peptide zone is followed by an unsaturated mono- or dicarboxylic acid (R^1, R^2), the dicarboxylic acids again being capped by the methyl esters of L-Arg, L-His, L-Lys. The mono- and dicarboxylic acids R^1 and R^2 all carry a C=C double bond. In the last step of the solid-phase ligand synthesis, this double bond is subjected to the Sharpless asymmetric dihydroxylation, using either AD-mix α or AD-mix β.[11,12] The diol substructure thus generated is intended to serve as a phosphoryl group acceptor.[4,5]

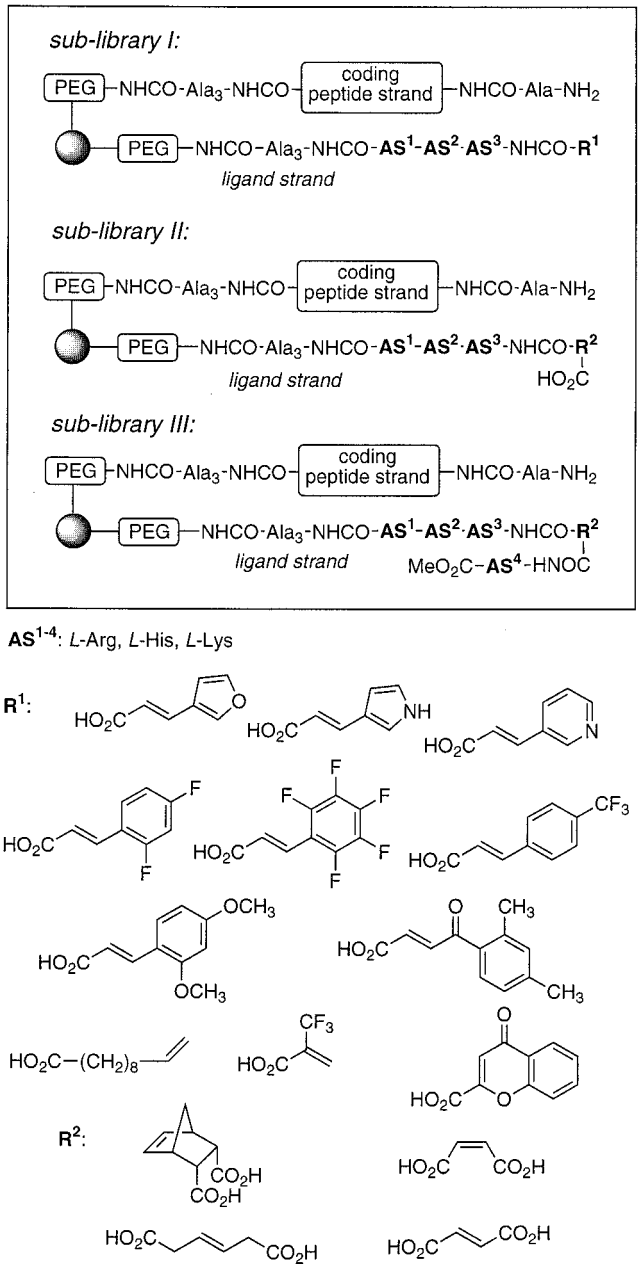

Figure 1. Structures of the building blocks AS^{1-4}, R^1, R^2 and the resulting ligand library. In the library, R^1 and R^2 represent the *gem*-diols obtained from the dihydroxylation of the olefinic mono- and diacids.

In the first step of the synthesis, TentaGel S-NH_2 was loaded with 3 × L-Ala as a spacer for the ligand strand and to facilitate and terminate the Edman degradation of the

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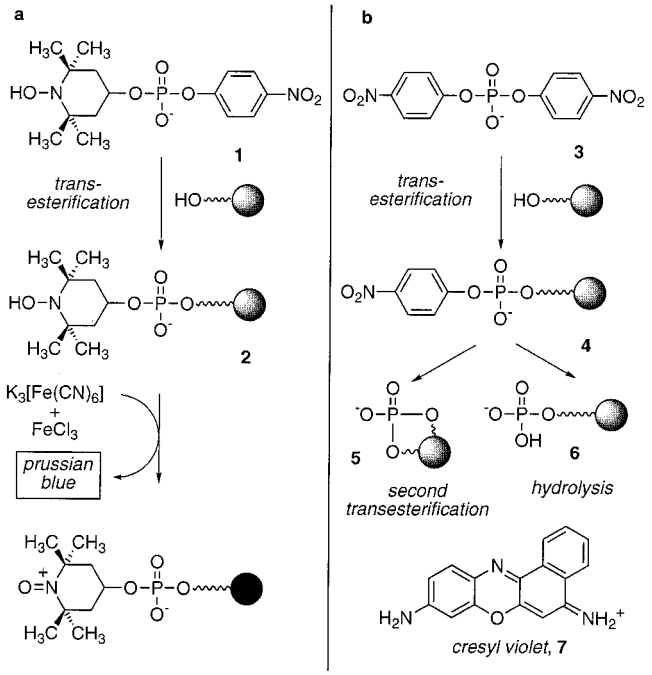

Figure 2. (a) Prussian blue assay for polymer bound phosphodiesterase activity; (b) ditto, cresyl violet assay.

coding strand. Then, L-Arg, L-His, and L-Lys were combinatorially placed in the following three positions. In all steps, a standard Fmoc/PyBOP protocol was followed.[13] Before adding R^1 or R^2, the corresponding coding amino acids were attached to ca. 10% of the resin's loading capacity. The polymer support was then saturated with R^1/R^2, again using PyBOP as the coupling agent. The yields of the Fmoc-amino acid additions were routinely ≥ 98%, and quantitative attachment of R^1/R^2 was indicated by a negative Kaiser test.[14] Addition of the unsaturated monoacids R^1 completed the ligand strand of the sublibrary I (Figure 1). When the four diacids R^2 were added, one part of the material was left as such (ligand strand of sublibrary II completed). L-Arg-OMe, L-His-OMe, or L-Lys-OMe was added to the other portion in a combinatorial manner, completing the ligand strand of sublibrary III. Finally, all polymer beads were pooled and subjected to the Sharpless dihydroxylation using AD-mix α or β. Of course, the nature of all 15 subunits R^1, R^2 was recorded in the coding strand, just as the type of AD-mix used. For the coding strand, only "nonfunctional" amino acids, e.g., Gly, Val, Phe (or combinations thereof), were used. The coding strand was finally capped with L-Ala, thus imbedding the coding amino acid sequence into a uniform "start signal" (1 × Ala) and a "stop signal" (3 × Ala) for the Edman analysis (Figure 1, see also Figure 4a,b).

The on-bead screening of the library for phosphodiesterase activity requires assays which allow for the optical detection of the locus of cleavage activity. Our approaches to this problem are summarized in Figure 2. Transesterification of the test substrate **1** attaches the hydroxylamine-phosphate unit to the ligand strand, affording **2**. In the subsequent treatment with $K_3[Fe(CN)_6]$ and $FeCl_3$, this hydroxylamine generates Fe^{2+}. Thus, insoluble prussian blue precipitates on the polymeric support (Figure 2a).

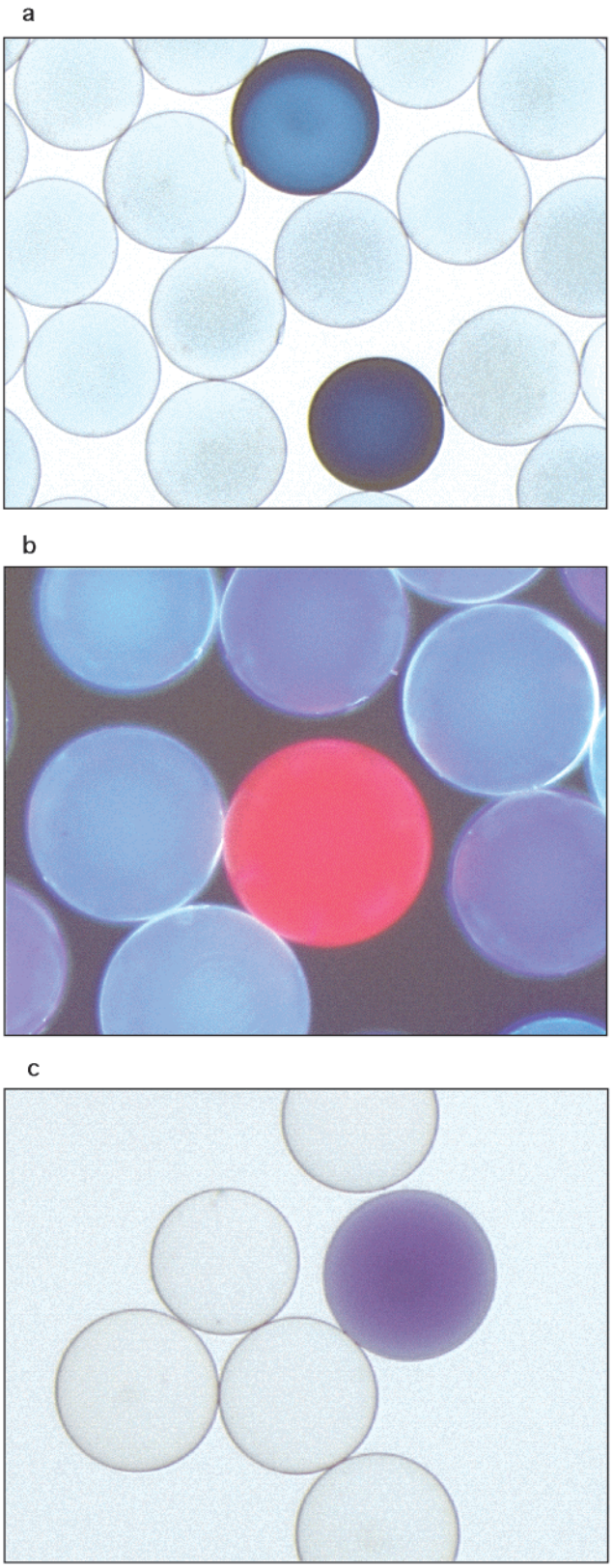

Figure 3. Screening of the library for phosphodiesterase activity: (a) prussian blue stain of beads carrying the ligand **8a**; (b) identification of a bead carrying the ligand **8b** by the cresyl violet assay (fluorescence); (c) ditto, absorption.

Alternatively, transesterification with bis(*para*-nitrophenyl)phosphate (**3**) leads to the phosphorylated products **4**−**6** (Figure 2b). Independent of the final stage of the transesterification, a phosphate group is transferred to the polymer

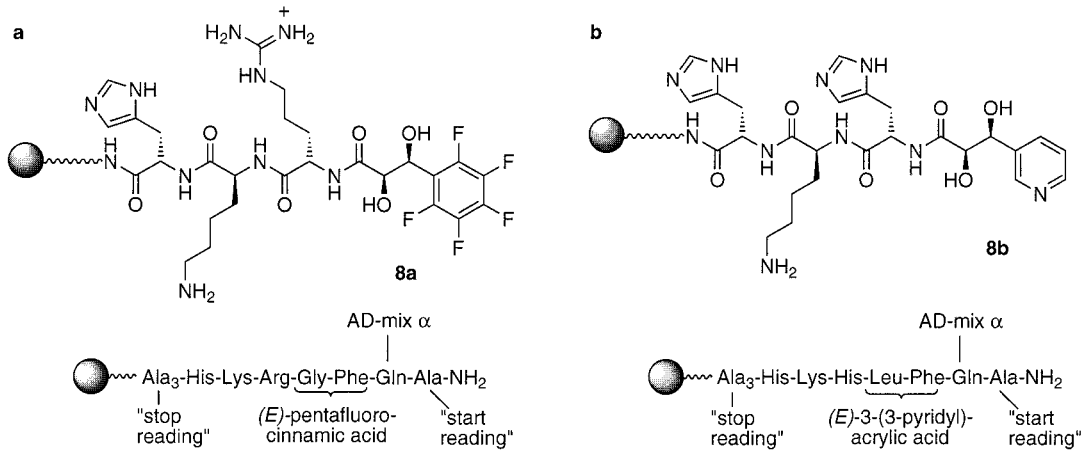

Figure 4. Full structures of the ligands **8a**,**b** and sequences of the coding strands.

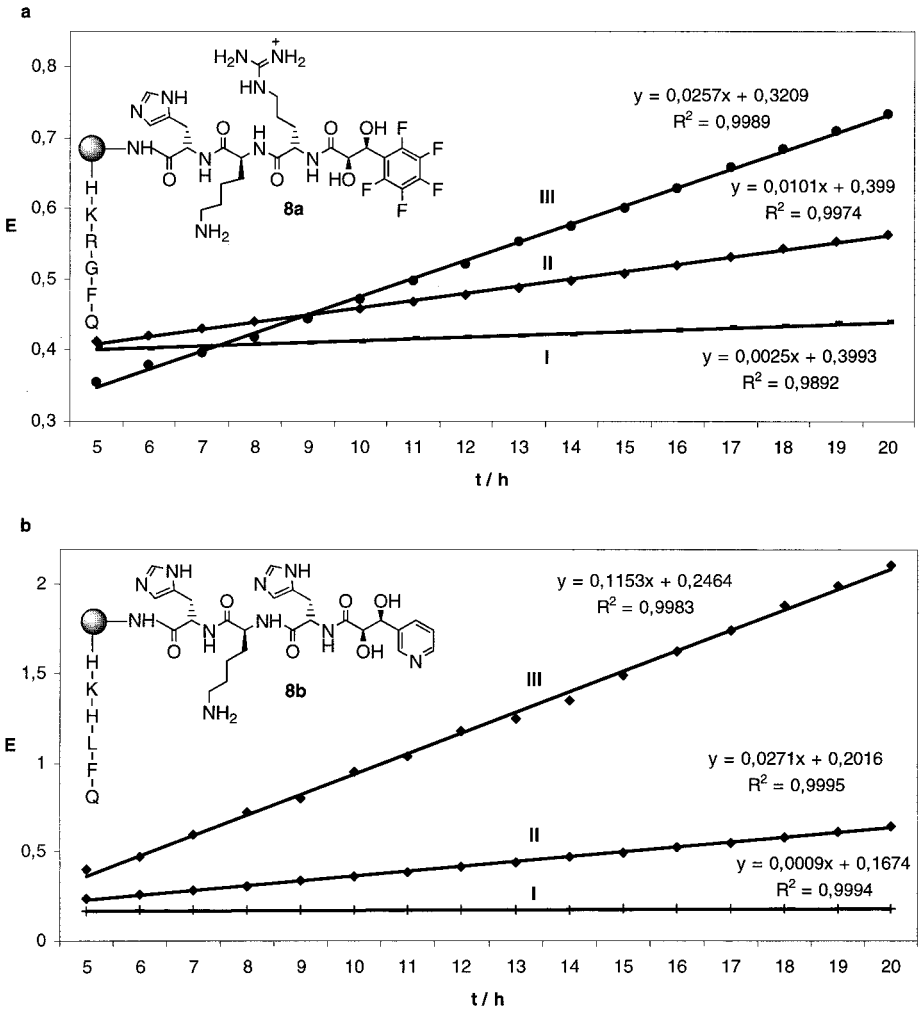

Figure 5. Liberation of *para*-nitrophenol from bis(*para*-nitrophenyl)phosphate (**3**, 20 °C, $\lambda_{obs} = 405$ nm): (a) Experiments using Zn^{2+} and ligand **8a** [EPPS-buffer (0.1 M, pH = 8), bis(*para*-nitrophenyl)phosphate (**3**, 5.0×10^{-3} M)]: trace I, buffer alone; trace II, buffer + $ZnCl_2$ (5.0×10^{-4} M); trace III, buffer + $ZnCl_2$ (5.0×10^{-4} M) + ligand **8a** (10^{-3} M). (b) Experiments using Eu^{3+} and ligand **8b** [MOPSO-buffer (0.1 M, pH = 7), bis(*para*-nitrophenyl)phosphate (**3**, 2.5×10^{-3} M)]: trace I, buffer alone; trace II, buffer + $EuCl_3$ (2.5×10^{-4} M); trace III, buffer + $EuCl_3$ (2.5×10^{-4} M) + ligand **8b** (10^{-3} M).

bead(s). This negatively charged phosphate group can be made visible by ion pairing with the cationic dye cresyl violet (**7**).

In the screening of the library, ca. 18 000 beads were incubated with the test substrate **1** and various metal ions at pH = 7 for 24 h at 20 °C (see Supporting Information for details). Figure 3a shows beads of the library after incubation with Zn^{2+} plus **1** and subsequent prussian blue assay. The most intensively stained bead was taken out and subjected to Edman degradation. The corresponding ligand **8a** was resynthesized on solid support, and its activity was cross-checked using the liberation of *para*-nitrophenol from bis-

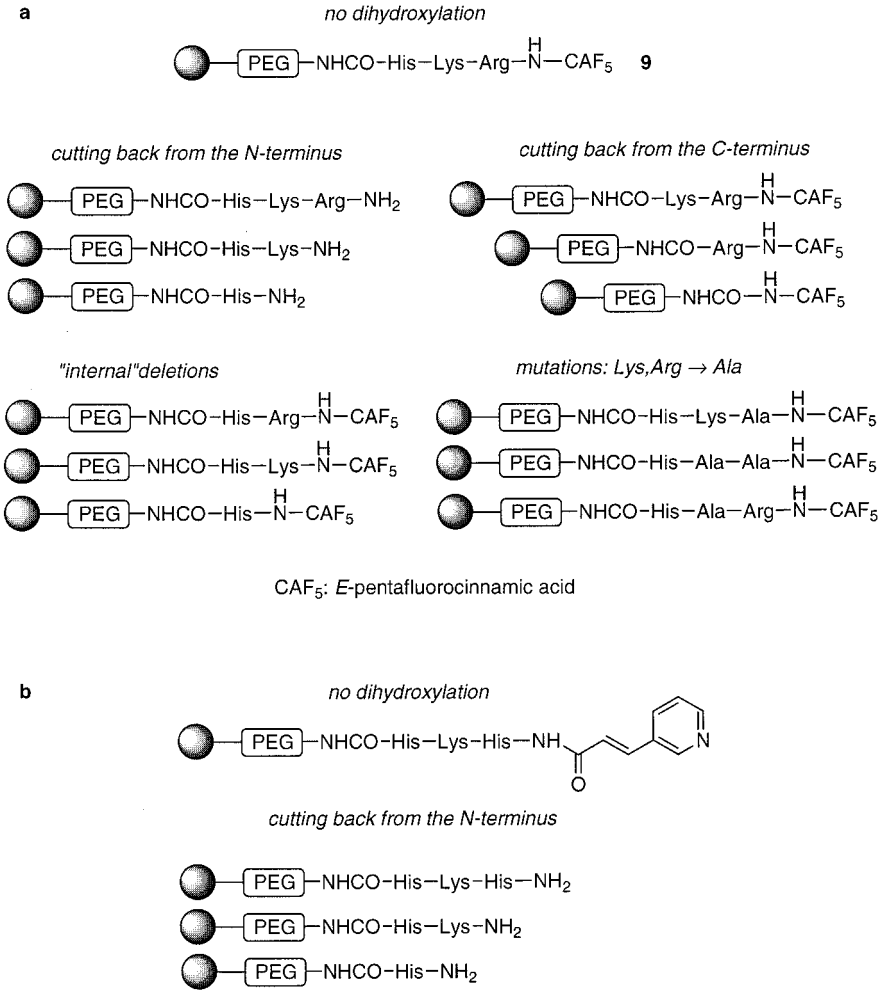

Figure 6. (a) Variations on the ligand **8a**; (b) variations on the ligand **8b**.

(*para*-nitrophenyl)phosphate (**3**): Indeed, the activity of the system composed of Zn^{2+} and the (polymer bound) ligand found in the Zn^{2+} assay could be confirmed. The full structure of the ligand **8a** is given in Figure 4a, together with the sequence of its coding strand. As shown in Figure 5a, the cleavage of bis(*para*-nitrophenyl)phosphate (**3**) proceeded ca. 2.5 times faster in the presence of the polymer bound ligand **8a** and Zn^{2+} compared to Zn^{2+} alone. In pure buffer, **3** is hydrolyzed ca. 10 times slower compared to the system composed of Zn^{2+} and the ligand **8a**.

Parts b and c of Figure 3 show positive beads in the assay using cresyl violet (**7**). The selective binding of cresyl violet (**7**) was nicely indicated by its blue color (Figure 3c) and its characteristic red fluorescence (Figure 3b). Decoding afforded the ligand structure **8b** (Figure 4b). Of course, no binding of cresyl violet (**7**) to the library beads was observed *prior* to incubation with bis(*para*-nitrophenyl)phosphate (**3**). As it turned out, the resynthesized polymer bound ligand **8b** showed best activity in combination with Eu^{3+}. In the presence of the polymer bound ligand **8b** and Eu^{3+}, the cleavage of bis(*para*-nitrophenyl)phosphate (**3**) proceeded ca. 4 times faster compared to Eu^{3+} alone and ca. 100 times faster compared to the background hydrolysis in the buffer system (Figure 5b). To the best of our knowledge, this is the largest ligand-induced acceleration of a phosphodiester cleavage effected by a mononuclear Eu^{3+} complex.[15,16]

For positive hits in a combinatorial library, it is usually assumed that the complete hit-structure is required for the response in a given assay. We were intrigued by the question of whether the complete ligand structures **8a**,**b**—polymer bound or in homogeneous solution − are required to effect the observed phosphodiester cleavage. Consequently, the ligands **8a**,**b** were dissected further. Figure 6a summarizes the structural variations carried out on the ligand **8a**. First, the *gem*-diol substructure was omitted, affording the unsaturated ligand **9**. As it turned out, **9** showed the same activity as the original system **8a**. All the truncations, deletions, and mutations shown in Figure 6a gave catalytically inactive material—no acceleration of the cleavage of bis(*para*-nitrophenyl) phosphate (**3**) beyond background could be observed. In other words: *All the structural elements of* ***9*** *are necessary for cleavage activity in combination with* Zn^{2+}. Finally, when the ligand **9** was resynthesized on Wang resin and cleaved off, the non polymer bound analogue of **9** did not show catalytic activity in the presence of Zn^{2+}. Thus, it must be concluded that the cleavage activity found here is a feature of the *properly structured and polymer bound ligand in combination with the right metal ion (i.e.,* Zn^{2+} *in the case of* ***9****).*

The variations/truncations carried out on the ligand **8b** are summarized in Figure 6b. We rapidly realized that *polymer bound histidine—in combination with* Eu^{3+}*—is the*

active substructure.[20] Furthermore, neither histidine itself, histidine amide, or histidine bound to poly(ethylene glycol) showed any rate acceleration relative to Eu^{3+} alone. In other words: the screening of the library with the cresyl violet assay identified a ligand that significantly enhances the phosphodiesterase activity of Eu^{3+}. However, contrary to the system composed of **8a**/Zn^{2+}, the assay actually responded to an active *substructure*. This most important point would have gone undetected without further dissecting the hit structure **8b**.

Conclusions

On the basis of our observation, a clear caveat must be inferred to the combinatorial search for new catalysts: As shown here, activity may well be generated de novo by combinatorial methods and identified by suitable assays. However, the characterization of a novel and active system should always include a thorough examination of the substructure−activity relation. In this respect, also the polymeric support must be considered as an important part of the structure in question.[18] We furthermore feel that (sub-)structure−activity analyses similar to the ones presented here may provide unexpected results also in the much broader field of combinatorial chemistry dealing with binding phenomena in general.[21,22]

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Supporting Information Available. Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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