Determination of the activity of heterofunctionalized catalysts from mixtures

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A conceptually new approach for the rapid determination of the activity of heterofunctionalized catalysts is described. A small library of catalysts is synthesized *via* a one-pot synthesis and screened for activity without separating the library members. The screening of libraries with varying catalyst distributions and subsequent deconvolution allows the determination of the activity of each catalyst separately. This approach is applied to a four-component library of biomimetic catalysts active in the cleavage of HPNP, a model compound for RNA. The rate constants for the four catalysts obtained *via* the deconvolution procedure are in very good agreement with the values obtained *via* the classical one catalyst–one screening approach. Simulations provide a hint of the scope and limitations of the approach.

Introduction

The design of artificial structures with multiple different functionalities is of strong current interest in the areas of molecular recognition and catalysis.[1] The presence of multiple different recognition/catalytic units in a heterofunctionalized molecular receptor allows a better tuning of its interaction with a substrate with respect to a homofunctionalized system. It is not surprising that in enzymes, which are the catalysts *par excellence*, heterofunctionalization in the recognition and catalytic sites is a rule.[2] The current strategy to address heterofunctionalized structures is based on the use of a molecular scaffold with the connector units masked by orthogonally compatible protective groups.[3] The desired functionalities are subsequently introduced in a stepwise manner *via* a series of deprotection–coupling steps. The disadvantage of this approach is the prerequisite of a multistep synthesis to first synthesize the protected scaffold molecule and next to introduce the different functional groups.

Here we propose a conceptually different approach to determine the activity of heterofunctionalized catalysts. Our approach is based on the one-step synthesis of small mixtures of catalysts, which are directly screened for catalytic activity without prior separation of the individual components. Consequently, the observed catalytic activity is a sum of the activity of each catalyst present in the mixture multiplied by its concentration.[4] Screening of mixtures with varying catalyst distributions, simply obtained by mixing the reagents in different ratios, and subsequent deconvolution allows the determination of the catalytic activity of each catalyst individually.[5]

In this report we show that with this protocol the catalytic activity of heterofunctionalized catalysts can be very rapidly determined with a minimum synthetic effort. To give a proof of principle, we have validated a minimal catalyst mixture (library) composed of four members. The catalytic activity of each member indirectly determined *via* the library method described above was compared to the values obtained *via* the classical one catalyst–one screening approach. This approach allows the determination of the activity of catalysts from mixtures, an area that is still largely unexplored, without using any sophisticated apparatus.[6,7]

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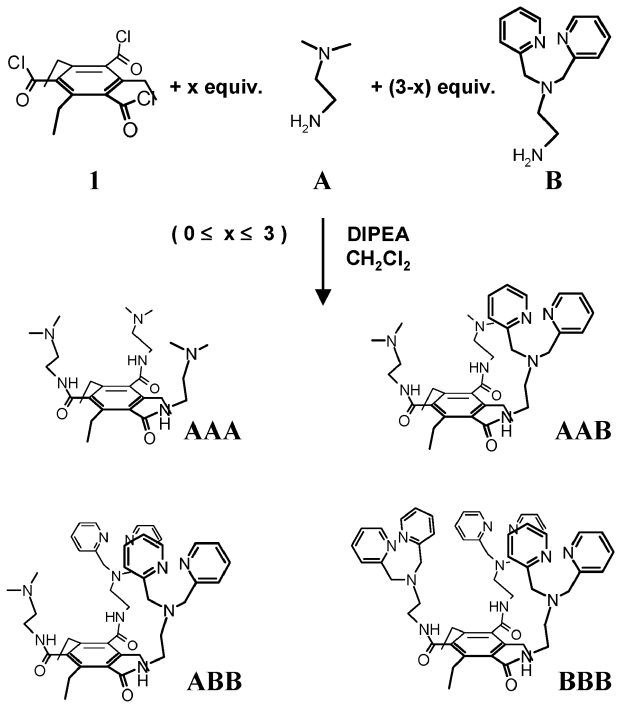
Results and discussion

Our long-standing interest in the design of artificial metallonucleases for the cleavage of the phosphodiester bonds of DNA and RNA prompted us to test our concept on catalysts able to cleave an RNA model substrate (HPNP, 2-hydroxypropyl-4-nitrophenylphosphate).[8] It is known that natural phosphodiesterases typically require for activity the cooperative action of two or more metal ions (often Zn^{2+}) possibly in conjunction with other functionalities acting as a general base or general acid.[9]

Therefore, simple catalysts providing a collection of such functionalities seemed very suitable to verify the proposed synthesis and screening approach. The catalysts are composed of a platform molecule **1** to which three functionalized arms are connected (Scheme 1). Scaffold **1** is a hexasubstituted benzene, where the adjacent substituents alternate up and down with respect to the benzene plane. This preorganizational effect of **1** has been widely used to create receptors and catalysts, most notably by Anslyn and co-workers.[10] As functional arms we have used *N*,*N*-dimethylethylenediamine, **A** and *N*,*N*-bis(2-pyridylmethyl)ethylenediamine, **B**. The basic dimethylamino group in arm **A** may serve in a general acid/base catalyzed reaction, whereas arm **B** serves as the metal binding site for Zn^{2+}.[11]

A one-pot reaction of scaffold **1** with a mixture of **A** and **B** gives a mixture of 4 potential catalysts **AAA**, **AAB**, **ABB**, and **BBB** with a distribution depending on the initial ratio of **A** and



Scheme 1 Synthetic procedure for the one-pot preparation of libraries of different composition.

B added to scaffold **1**. RP-HPLC analysis of the mixtures showed that exclusively the four catalysts are present in the mixtures, the peaks being assigned to each of them *via* ESI-MS spectrometry (representative HPLC spectra of some of the mixtures are given in Fig. 1). Within this series of mixtures, the distribution of the catalysts ranged from 75 : 24 : 1 : 0 to 0 : 11 : 61 : 28 for catalysts **AAA**, **AAB**, **ABB** and **BBB** respectively.[12] In order to validate the proposed screening method, pure samples of each of the four catalysts were isolated *via* preparative RP-HPLC.

Next, each mixture was screened for catalytic activity in the cleavage of HPNP. Reactions were performed in a buffered aqueous solution at pH 7.2 and 40 °C with an overall catalyst concentration of 20 μM and a 10-fold excess of substrate.[13] All

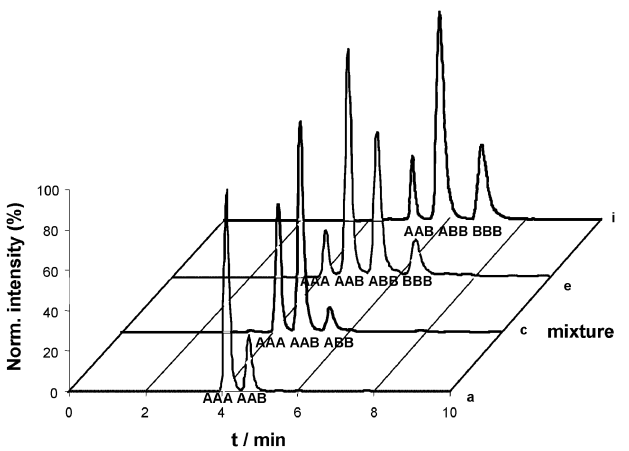


Fig. 1 HPLC elution profiles of four representative mixtures.

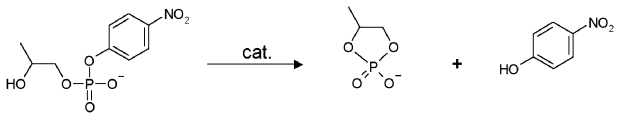
reactions were performed in the presence of 120 μM Zn^{2+} in order to ensure saturation of all metal binding sites deriving from arm **B**. Under these conditions the catalytic contribution of non-ligated Zn^{2+} has also to be considered.[14] For each mixture, the initial rate was determined *via* a linear regression over the first 10 000 s.

Independently, the catalytic activities of the pure catalysts **AAA**, **AAB**, **ABB**, **BBB** and Zn^{2+} were measured under identical conditions (Table 1). The observed rates for the catalyst mixtures are the sum of the rate contribution of each catalyst present in the mixture (including free Zn^{2+}) following: $v_{init} = k_{AAA}[\mathbf{AAA}] + k_{AAB}[\mathbf{AAB}] + k_{ABB}[\mathbf{ABB}] + k_{BBB}[\mathbf{BBB}] + k_{Zn}[Zn]$ in which v_{init} (M s^{-1}) is the experimentally observed rate and k (s^{-1}) the individual pseudo-first order rate constants for the different catalysts. Since the catalyst concentrations are known for each mixture, the individual rate constants (see Table 1) can be obtained *via* a simple deconvolution procedure. The procedure amounts to the determination of the coefficients (the rate constants, k) of n-component linear equations (with n being the number of catalysts present in the library). The minimum number of libraries of different composition is, hence, n. To minimize the error in our case we have analyzed libraries with nine different compositions instead of five (having four catalysts plus Zn^{2+}). The rate constants were determined in an iterative manner using the software Scientist.[15]

Comparison of the calculated values with the values obtained from direct measurement of pure catalysts **AAA**, **AAB**, **ABB** and **BBB**, shows, first of all, an excellent correlation which unequivocally validates our proposed screening method. Secondly, this comes at the cost of a relatively large error margin which is inversely related to the catalytic activity. Thus, the largest error is observed for compound **AAA** caused by a very low activity not exceeding 10 times the uncatalyzed reaction ($k_{uncat} = 3.9 \times 10^{-7}$ s^{-1})[8b] and therefore hardly affecting the reactivity profile. To address this point more generally, simulations of a library system were performed with varying catalytic activities of the library members. Artificial reactivity profiles were calculated for a simple reaction (first order both in substrate and catalyst) using the following rate equation, assuming identical catalyst mixture compositions as obtained by us experimentally: $d[P]/dt = k_1[R](f_{AAA}[\mathbf{AAA}] + f_{AAB}[\mathbf{AAB}] + f_{ABB}[\mathbf{ABB}] + f_{BBB}[\mathbf{BBB}])$ with [P] and [R] the product and reagent concentrations, respectively. Factors f_{AAA}–f_{BBB} were used to systematically modulate the catalytic activities of the four catalysts. Typically, a reactivity profile was generated for the 9 catalyst mixtures and, next, the obtained rates were taken as input values for the deconvolution procedure in order to calculate f_{AAA}–f_{BBB}. The factors thus obtained were compared to the input values. The results of three different simulations are given in Table 2.

Simulation **I** serves as a reference, with only catalyst **AAA** having catalytic activity. In this case, the reactivity profile is directly proportional to the relative amount of **AAA** present in the libraries. Deconvolution of the obtained reactivity profile gives accurately the right value for f_{AAA} with negligible values for the other catalysts. In simulation **II**, a situation is simulated where two good catalysts (**AAA** and **AAB**) are present ($f_{AAA} = f_{AAB} = 1000$). Also in this case, the fitting of the

Table 1 Observed rate constants (k_ψ) for the four catalysts in the cleavage of HPNP obtained both by measuring the activity of the pure catalysts and that of the mixtures



Catalyst	$10^5 k_\psi/s^{-1}$ [b]	$10^5 k_\psi/s^{-1}$ [c]	$10^5 k_\psi/s^{-1}$ [d]
AAA	0.4 (±0.1)	0.3 (±0.2)	—
AAB	0.9 (±0.2)	0.9 (±0.4)	0.9 (± 0.3)
ABB	2.2 (±0.4)	2.1 (±0.4)	2.1 (± 0.2)
BBB	5.1 (±0.2)	5.1 (±0.7)	—
Zn^{2+}	0.5 (±0.1)	0.5 (±0.2)	—

[a] Reaction conditions: [cat] = 20 μM, [HPNP] = 200 μM, [$Zn(NO_3)_2$] = 120 μM, [HEPES] = 10 mM, pH = 7.2, T = 40 °C. [b] From measurements of the pure components. [c] From measurements of the catalyst mixtures. [d] From measurements of the catalyst mixtures, fixing the homomeric species.

reactivity profile accurately provides the input values with minimal error. Finally, a situation is simulated (**III**) in which three species are catalytically active, but to a different extent (f_{AAA} = 1000, f_{AAB} = 100, f_{ABB} = 10) which probably resembles most closely a real experimental situation. In this case the simulation shows that the activities of **AAA** and **AAB** can be retrieved accurately with relatively small errors, whereas the contribution of the poorly performing catalyst **ABB** is not detected. This is obviously caused by the fact that the contribution of **ABB** is not significantly affecting the reactivity profile. These simple simulations seem to indicate that the ability to retrieve the rate constant for a certain catalyst is determined by the extent to which the catalyst contributes to the overall activity. In other words, the rate constants of catalysts that hardly outcompete the background reaction, in our experimental study for instance catalyst **AAA**, or that have a negligible activity with respect to another dominating catalyst present in the mixture, are difficult to determine with acceptable precision.

Not entirely satisfied with the large error margins obtained in the experimental study, especially for catalysts **AAA** and **AAB**, we decided to apply a more practical approach. In the catalyst library the only heteromeric catalysts are **AAB** and **ABB** whose synthesis would require the use of orthogonal protective groups. The other catalysts (**AAA**, **BBB**, and Zn^{2+}) can be obtained in pure form, and consequently, their activity can be studied separately. Therefore, the deconvolution procedure was repeated leaving only the rate constants for catalysts **AAB** and **ABB** as unknowns. To have less variables, we used only four catalyst mixtures instead of the previous nine. The obtained rate constants for the heteromeric catalysts are given in the third column of Table 1. Again, an excellent correlation was observed, but, importantly, this time also with error margins that are very acceptable for this kind of kinetic experiments, even for catalyst **AAB**.

Encouraged by this approach, we decided to investigate whether the analysis could be brought to a more sophisticated level. Repeating the same kinetics and deconvolution procedure at different substrate concentrations gives for each catalyst the observed rate constant k_{obs} as a function of the substrate concentration S. Since k_{obs} and S are related *via* $1/k_{obs} = (K_M/k_{cat})/S + 1/k_{cat}$, in this way the Michaelis–Menten parameters k_{cat} and K_M can be addressed for each catalyst. The obtained values for the heteromeric catalysts **AAB** and **ABB** are given in Table 3, together with the values obtained directly from measurements of the pure catalysts (**BBB** and Zn^{2+}). It should be noted that for compound **AAA** no saturation curve was obtained. The contribution of **AAA** therefore was ignored, with no apparent effect on the outcome of the deconvolution procedure.

Importantly, the values obtained for the heteromeric catalysts **AAB** and **ABB** *via* the deconvolution approach are in good agreement with the values obtained from the pure catalysts. The values are all within a 10% range, except for the k_{cat} value of **ABB** (20%). Considering the large intrinsic error which is common for this kind of kinetic measurement[8,9] (which is reflected by the relatively large error margins), and the fact that only 4 catalyst libraries were used for each

Table 2 Simulations of reactivity profiles

	f_{AAA}		f_{AAB}		f_{ABB}		f_{BBB}	
Simul.	In [a]	Out [b]	In	Out	In	Out	In	Out
I	1000	1000 (±9)	0	2 (±2)	0	14 (±4)	0	−3 (±9)
II	1000	994 (±1)	1000	1000 (±3)	0	6 (±4)	0	−3 (±2)
III	1000	1000 (±2)	100	110 (±5)	10	3 (±4)	0	3 (±4)

[a] In denotes the input value. [b] Out denotes the value obtained.

Table 3 Michaelis–Menten parameters for the heteromeric catalysts **AAB** and **ABB** obtained *via* measurement of the pure catalyst and *via* a deconvolution of the mixtures

Cat.	$10^4\ k_{cat}/s^{-1}$ [b]	$10^3\ K_M/M$ [b]	$10^4\ k_{cat}/s^{-1}$ [c]	$10^3\ K_M/M$ [c]
Zn^{2+}	0.3 (±0.1)	14.8 (±3.0)	—	—
AAB	2.4 (±0.4)	6.7 (±1.0)	2.5 (±0.4)	6.6 (±0.5)
BBB	6.4 (±0.9)	6.0 (±0.9)	5.8 (±0.8)	6.1 (±0.6)
BBB	10.7 (±0.2)	5.6 (±0.7)	—	—

[a] Reaction conditions: [cat] = 20 μM, [HPNP] from 0.1 to 2 mM, [$Zn(NO_3)_2$] = 120 μM, [HEPES] = 10 mM, pH = 7.2, T = 40 °C. [b] From measurements of the pure components. [c] From measurements of the catalyst mixtures.

substrate concentration, these results are very satisfactory. It is worth emphasizing that this approach allows the Michaelis–Menten parameters for **AAB** and **ABB** to be obtained without ever having the pure catalysts in hand.

Conclusions

In conclusion, we have reported a procedure to rapidly synthesize and screen heterofunctionalized catalysts. Catalyst preparation occurs in a one-step synthesis eliminating the use of any protective group. Screening for catalytic activity is directly performed on the mixtures without the need for separation. The rate constants for each individual catalyst are simply obtained by deconvoluting the overall rate constants of a series of mixtures with different catalyst distributions. The proof-of-principle study described here has validated the approach, but has also indicated some limitations in determining the activity of poorly performing catalysts. On the contrary, however, and of much more importance considering catalyst discovery, this method very accurately identifies the best catalyst for a given reaction. It is also worth pointing out that with this approach we have eliminated the frequently observed problem of false positive hits in combinatorial screening in cases where the high activity observed simply results from the combined activities of numerous weakly performing entities. In our opinion, these two advantages create a very high potential for this screening method. Currently, we are applying this protocol for the screening of much larger libraries.

Experimental

General procedure for the synthesis of the catalyst mixtures

To a solution of compound *N,N*-bis-(2-pyridylmethyl)ethylenediamine **B**[16] (3*x* equivalents with $x = 0.1–0.9$) and diisopropylethylamine (6 equiv.) in anhydrous CH_2Cl_2 (1 mL) was added a solution of platform **1** (25 mg, 0.072 mmol, quantitatively obtained by treating the corresponding triscarboxylic acid[17] with $SOCl_2$) in anhydrous CH_2Cl_2 (0.3 mL) and stirred for 90 min in a closed vial. Next, a solution of *N,N*-dimethylethylenediamine **A** [3(1 − *x*) + 2 equiv. with $x = 0.1–0.9$] and diisopropylethylamine (6 equiv.) was added and the reaction was stirred for 90 min after which an additional 2 equiv. were added. After stirring over night, volatiles were removed under reduced pressure and the residue was taken in CH_2Cl_2, washed with 1 M NaOH and brine, dried over Na_2SO_4 and evaporated to dryness.

HPLC analysis of the catalyst mixtures

Mixtures were analyzed using a Lichrosphere RP-C18 HPLC-column using H_2O–THF–TFA = 87.5 : 12 : 0.5 as the eluent. Peak detection occurred at 230 nm with peak integration directly yielding the ratios between the different components. The measured ratios obtained this way were verified independently using HPLC calibration curves of the separated catalysts which gave a maximal error margin of 5%.

Experimental data of the isolated four catalysts

Catalysts **AAA**, **AAB**, **ABB** and **BBB** were isolated using preparative RP-HPLC using a C18 column (5 μm). Purity was confirmed by analytical RP-HPLC.

2,4,6-Triethylbenzene-1,3,5-tris(*N,N*-dimethyl-ethylenediamine)-carboxyamide, AAA. ^{1}H NMR (250 MHz, $CDCl_3$) δ(ppm): 1.23 (t, $J = 7.5$ Hz, 9H, CH_2CH_3); 2.23 (s, 18H, $N(CH_3)_2$); 2.50 (t, $J = 5.8$ Hz, 6H, CH_2NH_2); 2.66 (q, $J = 7.5$ Hz, 6H, CH_2CH_3); 3.53 (m, 6H, CH_2 NHCO); 6.39 (m br, 3H, NH amide). ESI-MS (CH_3OH): *m*/*z* found 543.4, calc. 543.5 ($M + K^+$). HPLC (Lichrosphere RP-C18: H $_2$O–THF–TFA = 87.5 : 12 : 0.5): 4.1 min (100%).

2,4,6-Triethylbenzene-1,3-bis(*N,N*-dimethyl-ethylenediamine)-carboxyamide-5-(*N,N*-bis(2-pyridylmethyl)-ethylenediamine)-carboxyamide, AAB. ^{1}H NMR (300 MHz, CD_3CN) δ(ppm): 1.09 (t, $J = 7.5$ Hz, 6H, CH_2CH_3); 1.20 (t, $J = 7.5$ Hz, 3H, CH_2CH_3); 2.19 (s, 12H, $N(CH_3)_2$); 2.44 (t, $J = 6.3$ Hz, 4H, –CH_2–N of arm **A**); 2.53 (q, $J = 7.5$ Hz, 4H, CH_2CH_3); 2.65 (q, $J = 7.5$ Hz, 2H, CH_2CH_3); 2.81 (t, $J = 5.9$ Hz, 2H, –CH_2–N of arm **B**); 3.43 (m, 6H, CH_2NHCO); 3.85 (s, 4H, pyr–CH_2–N); 6.68 (br, 2H, NH amide **A**); 7.14 (m, 2H, 5-*H* pyr); 7.34 (d, $J^3 = 7.8$ Hz, 2H, 3-*H* pyr); 7.62 (m, 2H, 4-*H* pyr); 8.10 (br, 1H, NH amide **B**); 8.17 (d, $J^3 = 4$ Hz, 2H, 6-*H* pyr). ESI-MS (CH_3OH): *m*/*z* found 681.3 (100%), 697.4 (30%); calc. 681.4 ($M + Na^+$), 697.5 ($M + K^+$). HPLC (Lichrosphere RP-C18: H_2O–THF–TFA = 87.5 : 12 : 0.5): 4.9 min (100%).

2,4,6-Triethylbenzene-1-(*N,N*-dimethyl-ethylenediamine)-carboxyamide-3,5-bis(*N,N*-bis(2-pyridylmethyl)-ethylenediamine)-carboxyamide, ABB. ^{1}H NMR (300 MHz, CD_3CN) δ(ppm): 1.05 (t, $J = 7.5$ Hz, 3H, CH_2CH_3); 1.13 (t, $J = 7.5$ Hz, 6H, CH_2CH_3); 2.19 (s, 6H, $N(CH_3)_2$); 2.44 (t, $J = 6.3$ Hz, 2H, –CH_2–N of arm **A**); 2.57 (m, 6H, CH_2CH_3); 2.82 (t, $J = 5.8$ Hz, 4H, –CH_2–N of arm **B**); 3.45 (m, 6H, CH_2NHCO); 3.86 (s, 8H, pyr-CH_2–N); 6.68 (br, 1H, NH amide **A**); 7.11 (m, 4H, 5-*H* pyr); 7.34 (d, $J^3 = 7.8$ Hz, 4H, 3-*H* pyr); 7.62 (m, 4H, 4-*H* pyr); 8.12 (br, 2H, NH amide **B**); 8.19 (d, $J^3 = 4$ Hz, 4H, 6-*H* pyr). ESI-MS (CH_3OH): *m*/*z* found 835.4 (100%), 851.5 (30%); calc. 835.5 ($M + Na^+$), 851.6 ($M + K^+$). HPLC (Lichrosphere RP-C18: H_2O–THF–TFA = 87.5 : 12 : 0.5): 5.5 min (100%).

2,4,6-Triethylbenzene-1,3,5-tris(*N,N*-bis(2-pyridylmethyl)-ethylenediamine)-carboxyamide, BBB. ^{1}H NMR (300 MHz, DMSO-d_6) δ(ppm): 0.98 (t, $J = 7.2$ Hz, 9H, CH_2CH_3); 2.36 (q, $J = 7.2$ Hz, 6H, CH_2CH_3); 2.68 (t, $J = 6.4$ Hz, 6H, –CH_2–N of arms); 3.37 (m, 6H, CH_2NHCO); 3.80 (s, 12H, pyr-CH_2-N); 7.19 (m, 6H, 5-*H* pyr); 7.47 (d, $J^3 = 7.7$ Hz, 6H, 3-*H* pyr); 7.69 (m, 6H, 4-H pyr); 8.11 (br, 3H, NH amide); 8.39 (d, $J^3 = 4$ Hz, 6H, 6-*H* pyr). ESI-MS (CH_3OH): *m*/*z* found 989.8; calc. 989.5 ($M + Na^+$). HPLC (Lichrosphere RP-C18: H_2O–THF–TFA = 87.5 : 12 : 0.5): 6.7 min (100%).

General procedure for the screening of the catalytic activity

Cleavage experiments of HPNP were performed in H_2O buffered at pH 7.2 with HEPES (10 mM) at 40 °C. For solubility reasons the catalysts were added from a stock solution in 50% H_2O–CH_3CN. The catalyst concentrations

were calculated using an apparent molecular weight based on the measured HPLC distribution. Kinetic experiments were performed by measuring the absorbance of *p*-nitrophenolate at 400 nm at 2 min intervals for at least 3 h. After each measurement the pH was determined to exclude changes. Initial rates were calculated by linear regression over the first 10 000 seconds. Each measurement was performed at least in duplicate.

General procedure for deconvoluting the rates obtained for the catalyst mixtures

The initial rate is determined by the contribution of each catalyst present in the mixture following: $\nu_{init} = k_{AAA}[\mathbf{AAA}] + k_{AAB}[\mathbf{AAB}] + k_{ABB}[\mathbf{ABB}] + k_{BBB}[\mathbf{BBB}] + k_{Zn^{2+}}[Zn^{2+}]$. For each individual catalyst mixture the catalyst concentrations are known, leaving the rate constants as unknowns. Least square fitting was performed with Scientist[15] on nine mixtures contemporaneously.

Determination of the Michaelis–Menten parameters

Michaelis–Menten parameters were obtained for the pure catalysts under similar experimental conditions as before with substrate concentrations ranging from 0.1 to 3 mM (8 points). Initial rates were determined based on the first 1000 s and the plot of v_{init} *vs.* S was fitted using the Scientist package software with the Michaelis–Menten equation $v_{init} = V_{max} \times S/(K_M + S)$. Next, the Michaelis–Menten parameters for **AAB** and **ABB** were calculated by determining the observed rate constants k_{obs} for each of them at multiple substrate concentrations using the procedure identical as described before.

Acknowledgements

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References

1 (*a*) N. Srinivasan and J. D. Kilburn, *Curr. Opin. Chem. Biol.*, 2004, **8**, 305–310; (*b*) L. Baldini, A. J. Wilson, J. Hong and A. D. Hamilton, *J. Am. Chem. Soc.*, 2005, **126**, 5656–5657; (*c*) C. Chamorro and R. M. J. Liskamp, *J. Comb. Chem.*, 2003, **5**, 794–801; (*d*) S. C. McClesky, M. J. Griffin, S. E. Schneider, J. T. McDevitt and E. V. Anslyn, *J. Am. Chem. Soc.*, 2003, **125**, 1114–1115.

2 (*a*) A. Fersht, *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding*, W. H. Freeman, New York, 3rd edn, 1999; (*b*) A. J. Kirby, *Angew. Chem., Int. Ed. Engl.*, 1996, **35**, 707–724.

3 (*a*) M. B. Steffenson and E. E. Simanek, *Angew. Chem., Int. Ed.*, 2004, **43**, 5178–5180; (*b*) V. Del Amo, L. Siracusa, T. Markidis, B. Baragaña, K. M. Bhattarai, M. Galobardes, G. Naredo, M. N. Pérez-Payán and A. P. Davis, *Org. Biomol. Chem.*, 2004, **2**, 3320–3328; (*c*) A. Madder, L. Li, H. De Muynck, N. Farcy, D. Van Haver, F. Fant, G. Vanhoenacker, P. Sandra, A. P. Davis and P. J. De Clercq, *J. Comb. Chem.*, 2002, **4**, 552–562; (*d*) T. Opatz and R. M. J. Liskamp, *Org. Lett.*, 2001, **3**, 3499–3502; (*e*) X.-T. Zhou, A. Rehman, C. Li and P. B. Savage, *Org. Lett.*, 2000, **2**, 3015–3018.

4 Assuming an independent contribution from each catalyst.

5 A similar approach has been previously applied to supramolecular assemblies, but the dynamic nature of these systems has always prevented a validation of the results obtained for the heteromeric species. See: (*a*) M. T. Reetz, T. Sell, A. Meiswinkel and G. Mehler, *Angew. Chem., Int. Ed.*, 2003, **42**, 790–793; (*b*) P. Molenveld, J. F. J. Engbersen and D. N. Reinhoudt, *Angew. Chem., Int. Ed.*, 1999, **38**, 3189–3191.

6 K.-J. Johansson, M. R. M. Andreae, A. Berkessel and A. P. Davis, *Tetrahedron Lett.*, 2005, **46**, 3923–3926.

7 P. Chen, *Angew. Chem., Int. Ed.*, 2003, **42**, 2832–2847.

8 (*a*) F. Manea, F. Bodar Houillon, L. Pasquato and P. Scrimin, *Angew. Chem., Int. Ed.*, 2004, **43**, 6165–6169; (*b*) A. Scarso, U. Scheffer, M. Göbel, Q. B. Broxterman, B. Kaptein, F. Formaggio, C. Toniolo and P. Scrimin, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 5144–5149; (*c*) C. Sissi, P. Rossi, F. Felluga, F. Formaggio, M. Palumbo, P. Tecilla, C. Toniolo and P. Scrimin, *J. Am. Chem. Soc.*, 2001, **123**, 3169–3170; (*d*) P. Rossi, F. Felluga, P. Tecilla, F. Formaggio, M. Crisma, C. Toniolo and P. Scrimin, *Biopolymers*, 2000, **55**, 496–501.

9 (*a*) J. R. Morrow and O. Iranzo, *Curr. Opin. Chem. Biol.*, 2004, **8**, 192–200; (*b*) N. Williams, B. Takasaki, M. Wall and J. Chin, *Acc. Chem. Res.*, 1999, **32**, 485–493; (*c*) D. Wilcox, *Chem. Rev.*, 1996, **96**, 2435–2458.

10 (*a*) G. Hennrich and E. V. Anslyn, *Chem.–Eur. J.*, 2002, **8**, 2219–2224; (*b*) M. Komiyama, S. Kina, K. Matsumura, J. Sumaoka, S. Tobey, V. M. Lynch and E. V. Anslyn, *J. Am. Chem. Soc.*, 2002, **124**, 13731–13736.

11 (*a*) J. K. Romary, J. D. Barger and J. E. Bunds, *Inorg. Chem.*, 1968, **7**, 1142–1145; (*b*) A. Ojida, M.-A. Inoue, Y. Mito-oka and I. Hamachi, *J. Am. Chem. Soc.*, 2003, **125**, 10184–10185; (*c*) S. Takebayahi, M. Ikeda, M. Takeuchi and S. Shinkai, *Chem. Commun.*, 2004, 420–421.

12 An equal molecular absorption coefficient for the four catalysts at 230 nm was assumed, since at this wavelength the chromophore that differs the four species (pyridine) has a very low absorbance (ε = 393 L mol^{-1} cm^{-1}). As validation, the obtained concentrations were compared with concentrations obtained from calibration curves of the catalysts separately. The maximum error was 3%.

13 The average molecular weight of each mixture can be calculated from the mixture distribution.

14 The cleavage of HPNP was measured using UV-Vis spectroscopy following the increase in absorbance at 400 nm due to the release of the nitrophenolate ion.

15 MicroMath Scientist for Windows, version 2.01.

16 K. Hanaoka, K. Kikuchi, Y. Urano and T. Nagano, *J. Chem. Soc., Perkin Trans. 2*, 2001, 1840–1843.

17 S. V. Kolotuchin, P. A. Thiessen, E. E. Fenlon, S. R. Wilson, C. J. Loweth and S. C. Zimmerman, *Chem.–Eur. J.*, 1999, **5**, 2537–2547.