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# Supramolecular Tandem Enzyme Assays for Multiparameter Sensor Arrays and Enantiomeric Excess Determination of Amino Acids

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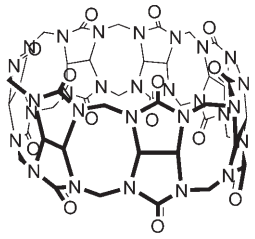
**Abstract:** The coupling of an enzymatic transformation with dynamic host-guest exchange allows the unselective binding of macrocycles to be used for highly selective analyte sensing. The resulting supramolecular tandem enzyme assays require the enzymatic substrate and its corresponding product to differ significantly in their affinity for macrocycles, for example, cation receptors, and to show a differential propensity to displace a fluorescent dye from its host-guest complex. The enzymatic transformation results in a concomitant dye displacement that can be accurately followed by optical spectroscopy, specifically fluorescence. By exploiting this label-free continuous enzyme assay principle with the fluorescent dye Dapoxyl and the macrocyclic host cucurbit[7]uril, a multiparameter sensor array has been designed, which is capable of detecting the presence of amino acids (e.g. histidine, arginine, lysine, and tyrosine) and their decarboxylases. Only in the presence of both, the particular amino acid and the corresponding decarboxylase, is the amine or diamine product formed. These products are more highly positively charged than the substrate, have a higher affinity for the macrocycle and, therefore, displace the dye from the complex. The extension of the high selectivity and μM sensitivity of the tandem assay principle has also allowed for the accurate measurement of D-lysine enantiomeric excesses of up to 99.98%, as only the L-enantiomer is accepted by the enzyme as a substrate and is converted to the product that is responsible for the observed fluorescence signal.

**Keywords:** amino acids · cucurbiturils · decarboxylases · fluorescence · host–guest systems

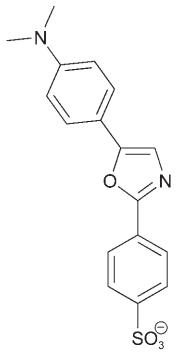
## Introduction

The inclusion of small guest molecules within larger macrocyclic hosts is a burgeoning field of research with a diverse range of applications, including biological examples ranging from analyte sensing[1–6] and drug delivery[7–9] to synthetic enzymes.[10,11] The combination of high affinity and selectivity, including enantioselective binding, has remained a constant challenge in host-guest chemistry, in which macrocycles have remained inferior to biological receptors, such as enzymes and antibodies.[12–15]

Cucurbiturils have recently emerged as an interesting class of macrocycles with a high affinity for neutral and cationic organic guests,[16–33] and in a few exemplary instances, with host-guest binding constants approaching that of biotin-avidin.[25,26,29] However, cucurbiturils, for example, cucurbit[7]uril (CB7, composed of 7 glycoluril units), suffer from the same shortcoming of other macrocycles in that their overall selectivity is mediocre in the context of biomimetic applications. For example, they bind—in their function as cation receptors—aliphatic amines and diamines in their ammonium forms strongly,[20–25] but they do so with low selectivity, that is, with similar binding constants for different organic residues and for differently sized cucurbiturils. Moreover, although a chiral cucurbituril derivative has



cucurbit[7]uril (CB7)



Dapoxyl

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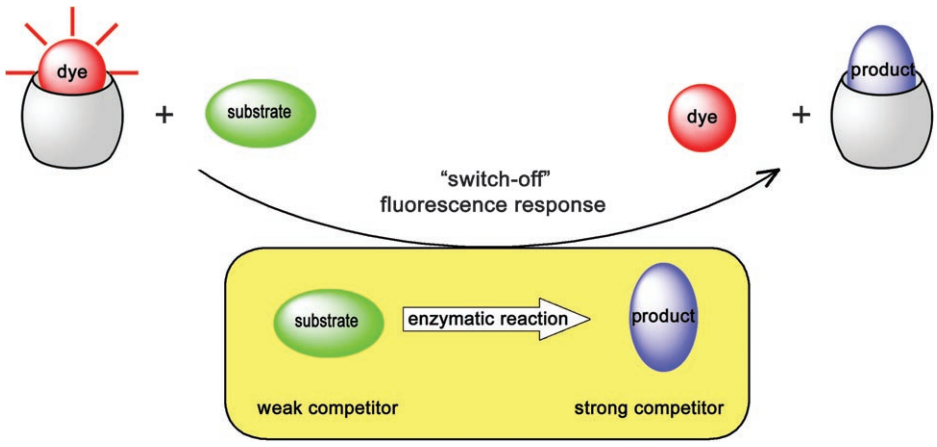
recently been documented,[34] the parent cucurbiturils are achiral and, therefore, tend to be unsuitable for enantioselective binding unless a chiral template is present.[34–36] Herein, we combine the generality of cucurbituril encapsulation with the selectivity and enantiospecificity of enzymatic reactions and demonstrate how the unselective binding of macrocycles can be exploited for highly sensitive, selective, and even enantioselective recognition of analytes.

The analyte sensing (Scheme 1) employs an indicator displacement strategy[37–40] according to the supramolecular tandem assay principle that we have recently communicated for monitoring enzymatic activity.[41] The working principle is based on a competition between a fluorescent dye and the product of the enzymatic reaction for cucurbituril encapsulation. The enzymatic decarboxylation of the amino acid converts a weak competitor to a more highly positively charged diamine (strong competitor) which leads to a successive displacement of the fluorescent dye from the complex in the course of the enzymatic reaction. Accordingly, the fluorescent dye is suitably selected such that it shows a large change in fluorescence in its complexed and uncomplexed forms; several dyes with these desirable properties have already been reported for cucurbiturils,[17,42–45] with Dapoxyl as a fluorescent dye of choice to use with CB7 near the physiological pH ideal for enzymatic activity. Dapoxyl shows an up to 200-times stronger fluorescence intensity in its CB7 complex,[17] such that the addition or enzymatic formation of a competitor leads to a fluorescence decrease ("switch-off"). CB7 and Dapoxyl form the so-called reporter pair of the tandem assay. Herein, we use this reporter pair to document two applications of tandem assays that, while easy to implement, are very powerful analytical tools. First, we construct a multiparameter sensor array capable of detecting either the presence of a particular amino acid or, vice versa, its respective decarboxylase. Second, we apply the supramolecular tandem assay to the determination of the enantiomeric excess of mixtures of D- and L-amino acids within an extremely high *ee* (*ee* = enantiomeric excess) range that is inaccessible to conventional analytical techniques.




Scheme 1. Working principle of a "switch-off" product-coupled tandem assay.

## Results and Discussion

**Development of a multiparameter sensor array**: Amino acid decarboxylases play an important role in many diverse biochemical pathways in both plants and animals, such as immune regulation, vasodilatation, smooth muscle contraction, gastric acid secretion, allergic response, and wound healing in animals as well as cell proliferation and differentiation, morphogenesis, dormancy and germination, tuberization, flower induction and development, embryogenesis, fruit-set and growth, and fruit ripening in plants.[46,47] Most decarboxylases are specific to single amino acids, whereas others (such as DOPA decarboxylase) are known to react with classes of amino acids, for example those with aromatic residues.[48,49] Decarboxylases are ideal for investigation by means of supramolecular tandem assays, because their reaction leads to a large variation of charge status between the substrate and product. This has a similarly large influence on the differential binding, and, consequently, the fluorescence response according to Scheme 1.[41] In advancing a sensor application, we aimed to exploit the nonselectivity of the macrocyclic receptor CB7 which should allow for the sensing of several amino acids through their specific reactions with decarboxylases.

We selected four amino acids with their associated specific amino acid decarboxylases for investigation: histidine, arginine, lysine, and tyrosine. The amino acids ornithine and tryptophan were included as additional test cases, although we lacked access to the corresponding decarboxylases. The essential first step in advancing a tandem assay is to determine the binding constants ($K_C$) of both potential competitors (substrate and product), because these must show a significant difference (factor 10 or larger) for the assay principle to be practically useful. As quantified by both isothermal titration calorimetry (ITC, see Figure 1) and competitive fluorescence titrations employing Dapoxyl as an indicator fluorescent dye (Figure 2),[41] the amino acids investigated in Table 1 all have a much lower (2–4 orders of magnitude) affinity to CB7 than their decarboxylation products. In general, the binding of the amines is enthalpically more favorable than that of the corresponding amino acids, but for the aliphatic diamines (cadaverine, putrescine) there is also a large entropic driving force. The latter is presumed to have a sizable contribution due to desolvation, which has been previously discussed for diamine binding to the smaller homologue cucurbit[6]uril.[22,50]

In keeping with our preliminary results,[41] the addition of either histidine, arginine, lysine, or tyrosine decarboxylase to the respective amino acid in the presence of Dapoxyl (2.5 μM)

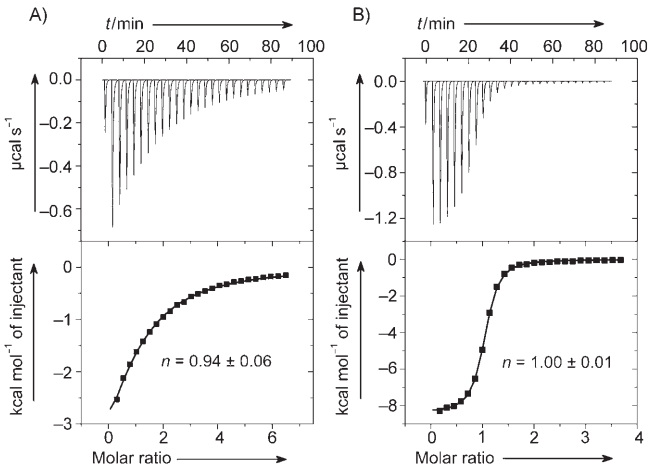



Figure 1. Calorimetric titrations of CB7 with A) the amino acid tyrosine and B) its decarboxylated product tyramine. Experiments were performed at 30 °C in 10 mM ammonium acetate buffer (pH 6.0) with the following concentrations: A) 1 mM of tyrosine, titrated into 0.03 mM of CB7 and B) 0.5 mM of tyramine titrated into 0.03 mM CB7. The top plots show the instrumental power function versus time (injected aliquots). The bottom plots show the heats of reaction obtained from the integration of the calorimetric traces, plotted against the amino acid/CB7 molar ratio.

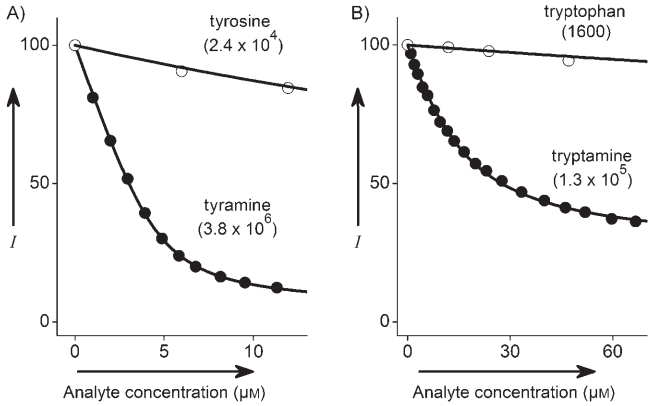



Figure 2. Competitive fluorescence titrations ($\lambda_{exc}=336$, $\lambda_{obs}=380$ nm) of the amino acids A) tyrosine and B) tryptophan and their respective decarboxylation products with competitive displacement of Dapoxyl (2.5 μM) from CB7 (10 μM) in 10 mM $NH_4OAc$ buffer (pH 6.0). The binding constants determined from the titrations ($K_C$, 10% error, cf. the Experimental Section) are given in parentheses. The titration plots for tyrosine and tryptophan extend to higher concentrations which is not shown in this truncated representation. Note that none of the investigated amino acids shows any absorbance at the selected excitation wavelength.

and CB7 (10 μM) maintained in a 10 mM $NH_4OAc$ buffer (pH 6.0) led to the expected decrease in fluorescence intensity (switch-off fluorescence response, Figure 3).[51] As can be seen for the sizable binding constant of tyrosine (Table 1), a weak affinity of the substrate is no prerequisite for applying the supramolecular tandem assay principle. Rather, as already emphasized in the preliminary study, it is the differential affinity, that is, the increased affinity for tyramine, which is sufficient to generate a readily detectable fluorescence response.

Previously, we have exploited this fluorescence response to detect enzymatic activity, determine enzyme kinetics, and suggested their use for inhibitor screening.[41] Herein, we employ the assays for the first time for selective sensing. Two sets of four solutions containing identical enzyme, amino acid, and reporter pair concentrations were prepared. In the first set, each preparation contained one of the four decarboxylases and the three amino acids that the decarboxylase was (supposedly) inactive against, whereas in the second set, the preparations contained all four amino acids (Figure 3). Confirming the specificity of the enzymatic transformations, it was observed that no experimentally significant fluorescence response was observed in the attempted decarboxylation of the incorrect amino acids. Note that the overall selectivity of the macrocycle with respect to either the four selected amino acids or their enzymatic products (histamine, agmatine, cadaverine, tyramine) is low, with the binding constants differing by less than three orders of magnitude for both sets (Table 1). This selectivity is, however, ir-

Table 1. Stability constants ($K_c$) and thermodynamic values for the complex formation between CB7 as host with amino acids and their decarboxylated products as guests.

| Guest | $K_C$ [$M^{-1}$] | | $\Delta G$ [kcal mol$^{-1}$] | $\Delta H$ [kcal mol$^{-1}$] | $T\Delta S$ [kcal mol$^{-1}$] |
|---|---|---|---|---|---|
| | Fluorescence[a] | ITC[b] | | | |
| histidine | 400 | –[c] | – | – | – |
| histamine | $3.2\times10^{4}$[d] | $(1.8\pm0.3)\times10^{4}$ | $-5.9\pm0.1$ | $-2.3\pm0.1$ | $3.6\pm0.1$ |
| arginine | 310 | $327\pm16$ | $-3.4\pm0.1$ | $-1.2\pm0.1$ | $2.2\pm0.1$ |
| agmatine | $1.1\times10^{6}$ | $(7.8\pm0.6)\times10^{5}$ | $-8.2\pm0.1$ | $-4.9\pm0.2$ | $3.3\pm0.3$ |
| lysine | 870 | $800\pm60$ | $-5.3\pm0.1$ | $-4.1\pm0.1$ | $-1.2\pm0.1$ |
| cadaverine | $1.4\times10^{7}$ | $(4.3\pm0.9)\times10^{6}$ | $-9.2\pm0.1$ | $-4.1\pm0.7$ | $5.1\pm0.8$ |
| tyrosine | $2.4\times10^{4}$ | $(2.2\pm0.3)\times10^{4}$ | $-5.9\pm0.1$ | $-6.5\pm0.6$ | $-0.6\pm0.5$ |
| tyramine | $3.8\times10^{6}$ | $(2.3\pm0.1)\times10^{6}$ | $-8.7\pm0.1$ | $-8.5\pm0.1$ | $0.2\pm0.1$ |
| tryptophan | 1600 | $(1.9\pm0.1)\times10^{3}$ | $-4.5\pm0.1$ | $-6.0\pm0.1$ | $-1.5\pm0.1$ |
| tryptamine | $1.3\times10^{5}$ | $(4.7\pm0.1)\times10^{4}$ | $-6.5\pm0.1$ | $-8.3\pm0.4$ | $-1.8\pm0.4$ |
| ornithine | 380 | –[c] | – | – | – |
| putrescine | $3.7\times10^{5}$ | $(3.0\pm0.1)\times10^{5}$ | $-7.6\pm0.1$ | $-3.3\pm0.1$ | $4.3\pm0.1$ |

[a] From competitive fluorescence titrations at 25 °C in 10 mM ammonium acetate buffer, pH 6.0, cf. reference [41]; 10% error. [b] Mean values measured from at least three experiments at 30 °C in 10 mM ammonium acetate buffer, pH 6.0; error given as standard deviation ($\pm1\sigma$). [c] Measured heat effects were too small in comparison to the dilution effect to allow an accurate determination of stability constants and reaction enthalpies. [d] Measured in 10 mM sodium phosphate buffer, pH 7.4 at 30 °C.

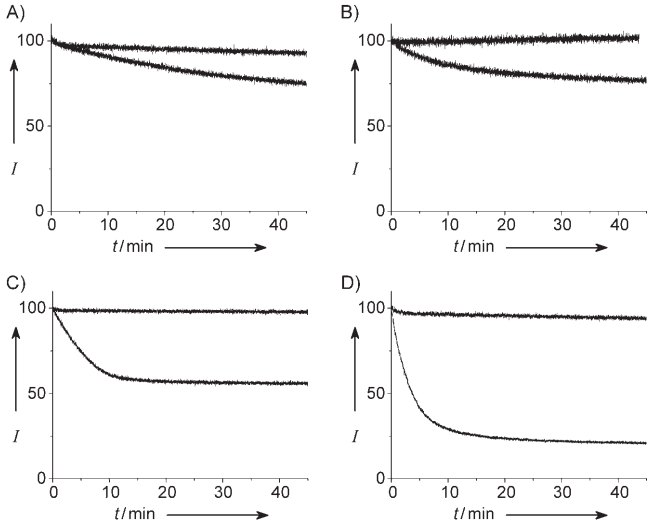



Figure 3. Fluorescence monitoring ($\lambda_{exc}=336$, $\lambda_{obs}=380$ nm, cuvette format) of the decarboxylation of four L-amino acids: A) histidine, B) arginine, C) lysine, D) tyrosine. In each instance the approximately constant fluorescence intensity refers to the sample that contained only the three amino acids the decarboxylase was inactive towards, whereas the time-resolved decrease in fluorescence refers to the solutions containing also the correct amino acid. The 10 mM $NH_4OAc$ buffer solutions (pH 6.0) contained 2.5 μM Dapoxyl, 10 μM cucurbit[7]uril, 40 μg mL$^{-1}$ of the appropriate amino acid decarboxylase, and 50 μM of the appropriate amino acids histidine, arginine, lysine, and tyrosine.[51]

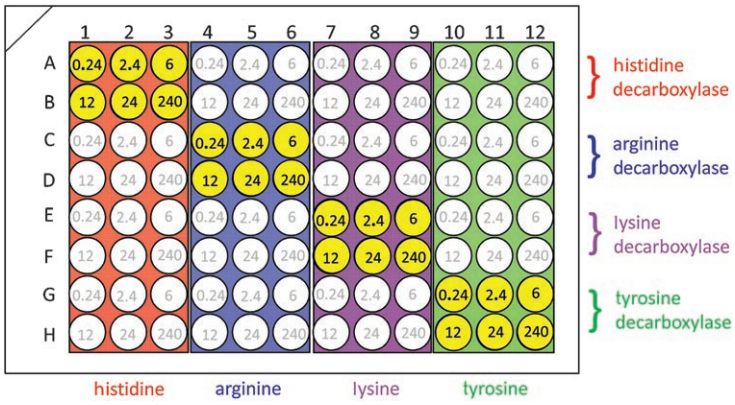



Figure 4. Microtiter plate setup employed for the multiparameter sensor array. The numbers within the wells refer to the amount of amino acid present, in nanomoles. Yellow wells mark expected positive responses.

relevant for the selective sensing, because it is the presence of the decarboxylase alone that ensures the selectivity of analyte sensing and the fluorescence response. In other words, a target amino acid analyte will interact with the host-dye reporter pair only in the presence of the complementary enzyme, because only in this case will the product be formed, which is responsible for the displacement of the dye.

In further detail, a comparison of the enzyme kinetics in the solutions containing all four amino acids was identical, within error, to solutions containing only the enzyme and the designated target (data not shown). This result showed that the presence of other amino acids did not interfere with the actual enzymatic reaction and, in particular, that they did not display any inhibitory effects. Consequently, CB7 can be used for the highly selective sensing of amino acids. Regarding the sensitivity, concentrations of 1 μM were readily detectable in cuvette-based measurements, that is, gave rise to a sizable fluorescence response.

We have also employed 96-well microtiter plate measurements to miniaturize the detection system to allow the design of a multiparameter sensor array, capable of screening several analytes (amino acids) in parallel. To scrutinize the sensitivity and explore the working range with respect to the analyte concentration, we prepared solutions of the four amino acids in six different amounts (ranging from 0.24 to 240 nmoles per well) with each of the four associated decarboxylases for a total of 96 solutions (see Figure 4).[51] As three out of every four solutions were exposed to the incorrect decarboxylase, there were only 24 possible positive responses per plate, allowing the other samples to be used as negative controls. An example at a particular well concentration (12 nmoles) is shown in Figure 5, which nicely illustrates how the 4 positive reactions (e.g., histidine in the presence of histidine decarboxylase) can be immediately differentiated from the 12 negative controls lacking the right combination. It is noteworthy, that by taking fluorescence intensity measurements at set intervals, we could monitor the decrease in fluorescence over time as a measure of the progress of the enzymatic reaction. Such time-dependent measurements provide an additional form of internal control, as any external sources of fluorescence that do not directly interact with the reporter-pair will be *static* in nature and would not affect the determinations. Static fluorescence can be easily differentiated from the time-resolved *change* in fluorescence due to the enzymatic reaction. This could become especially advantageous when applied to complex

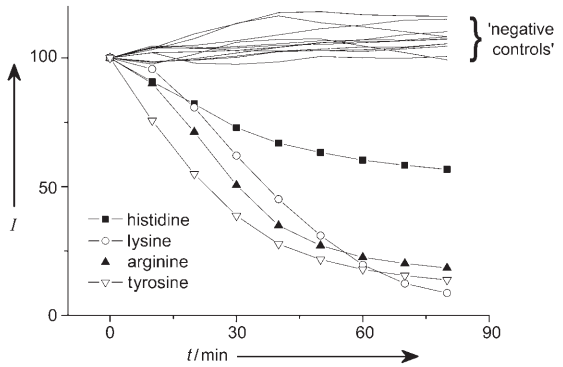



Figure 5. Time-dependent fluorescence responses for the sensing of L-amino acids by means of a supramolecular tandem assay system in a 96-well microtiter plate format (240 μL). Shown are the fluorescence responses for the L-amino acids tested at 12 nmoles per well in the absence (negative controls, top) and presence of the correct decarboxylase (lower four traces). The 10 mM $NH_4OAc$ buffer (pH 6.0) solutions contained 7.5 μM Dapoxyl, 30 μM CB7, and either 40 μg mL$^{-1}$ (lysine and tyrosine), 80 μg mL$^{-1}$ (arginine), or 160 μg mL$^{-1}$ (histidine) decarboxylase added at $t=0$.[51,52]

matrices that may contain multiple sources of interfering fluorescence.

Figure 6 shows the fluorescence decays obtained from the microtiter plate experiments at different lysine concentrations. The shaded region is the average response from the

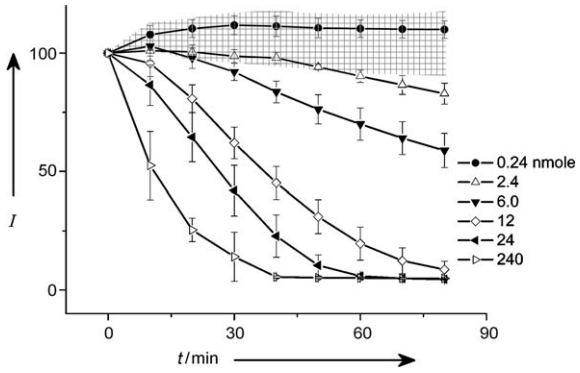



Figure 6. Time-dependent fluorescence response for the sensing of L-lysine by means of a supramolecular tandem assay system in a 96-well microtiter plate format (240 μL). Shown are the fluorescence responses for different L-lysine concentrations ranging from 0.24 to 240 nmoles per well (cf. legend). The patterned region is the average response from the negative controls (±2σ) lacking the correct decarboxylase. The 10 mM $NH_4OAc$ buffer (pH 6.0) solutions contained 7.5 μM Dapoxyl, 30 μM CB7, and 40 μg mL$^{-1}$ lysine decarboxylase added at $t=0$. Data were recorded in a 96-well microtiter plate with a sample volume of 240 μL.[51,52]

negative controls (±2σ).[52] As can be seen, the detection limit decreases with increasing measurement time and lies around 2.5 nmole per well after 60 min. This corresponds to analyte concentrations of 10 μM (240 μL well), which lies expectedly above the detection limit in the more sensitive cuvette-based spectrofluorometer measurements (1 μM, 1 mL, see above). Increasing substrate concentrations gave rise to faster reaction rates, as reflected in steeper fluorescence decays. Measurement of the reaction rate during the initial phase of reaction (Figures 5 and 6) could principally allow a semi-quantitative amino acid sensing, which was explored in further detail in the context of the *ee* determination (see below). For qualitative detection, an initial measurement and a second one at a fixed incubation time is principally sufficient; this mode would be preferable for automated detection, that is, for high-throughput screening, environmental monitoring, or quality control.

**Determination of enantiomeric excess**: The synthesis of D-amino acids is becoming increasingly important due to numerous applications ranging from antibiotics and anticoagulants to pesticides and fertility drugs.[53] Accordingly, there is also an increased interest in the preparation and isolation of these D-enantiomers,[53] and, in particular, in the determination of their optical purity.[54] The method that we developed for the sensing of amino acids (see above) is specific for the natural L forms, and the accurate determination of their absolute concentration in the presence of a very large excess of the respective D form should provide a viable analytical method to determine the *ee* of the latter. A related method for *ee* determination based on the absolute concentration of only one enantiomer has recently been described by Matile and co-workers in the enantiospecific enzymatic degradation of polymeric analytes, such as poly-L- versus poly-D-glutamate, in which the selective formation of monomeric L-glutamate results in blockage of a synthetic pore in dependence on the *ee* of the polymer.[55] The method allowed *ee* determinations between 90–98%, which have proven difficult to assess by conventional analytical techniques, such as CD, GC, or HPLC. Another example of a related determination of *ee* involves the enantiospecific enzymatic oxidation of (*S*)-1-phenylpropanol,[56] in which the formation of the reduced cofactor NADPH, followed spectrophotometrically, provided indirect information about the optical purity of the alcohol with an accuracy of ±10% *ee*.

To demonstrate the viability of our envisaged method, we selected the reaction of L-lysine with its respective decarboxylase. As demonstrated above, the decarboxylation results in a readily measurable fluorescence decrease, the rate of which depends on the absolute L-amino acid concentration. The enantiospecificity of lysine decarboxylase was demonstrated through an attempted decarboxylation of a commercial D-lysine sample (up to 1 mM) under identical reaction conditions (10 μM CB7, 2.5 μM Dapoxyl, and 40 μg mL$^{-1}$ lysine decarboxylase, maintained in 10 mM $NH_4OAc$ buffer adjusted to pH 6.0). No temporal change in fluorescence intensity was observed upon addition of the enzyme (up to 45 min), confirming, as expected, that the enzyme did not affect decarboxylation of this enantiomer. Consequently, it appeared promising to assess the concentration of L-lysine even in the presence of a large excess of D-lysine and thereby determine the *ee* of such mixtures.

In the initial *ee* determinations, a first series of solutions was prepared that contained 50 μM L-/D-lysine in total. 1–9 μM of those were L-lysine, which could be reliably detected in a cuvette format (see above), and the remainder (41–49 μM) was D-lysine. These relative concentrations correspond to an *ee* of D-lysine ranging from 64–96%; a control experiment with an *ee* of 100% (pure D-form) is also included in Figure 7. Figure 7 shows the corresponding kinetic fluorescence decay traces, which were approximately linear in the initial time range (up to 20 min) and showed a slight positive curvature only at higher L-amino acid concentrations and reaction times. The fluorescence decay kinetics were dependent on the substrate concentration, and we determined the initial rates from the slopes to obtain a measure of the absolute concentration of the reactive L form. This was arbitrarily done in the fixed time interval between 10 and 20 min, as small initial fluctuations were occasionally observed directly after addition of enzyme, presumably due to mixing and temperature equilibration. The resulting initial rates showed a linear dependence on the concentration of L-lysine ($r=0.997$, Figure 8A), consistent with the characteristic enzyme kinetics at substrate concentrations far below the $K_m$ value (300 μM for lysine decarboxylase).[41,57] A similar linear relationship ($r=0.957$, Figure 8B) was ob-

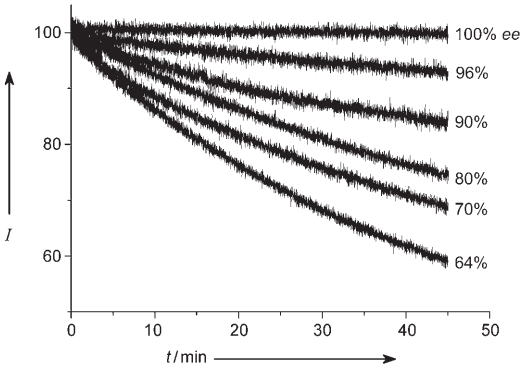



Figure 7. Time-dependent fluorescence response ($\lambda_{exc}=336$, $\lambda_{obs}=380$ nm, cuvette format) monitoring L-lysine decarboxylation with varying D-lysine enantiomeric excess. The 10 mM $NH_4OAc$ buffer (pH 6.0) solutions contained 2.5 μM Dapoxyl, 10 μM CB7, and 40 $\mu g mL^{-1}$ lysine decarboxylase added at $t=0$. The combined D/L-lysine concentration was 50 μM.

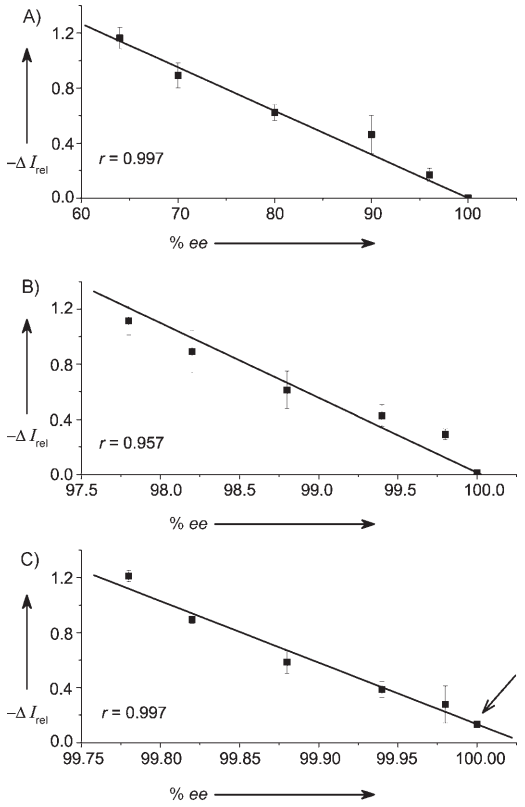



Figure 8. Linear relationship between the *ee* of D-lysine and the reaction rate, shown for the three ranges of enantiomeric excess studied: A) 60–96%, B) 97.8–99.8%, C) 99.78–99.98% (correlation coefficients are shown in the bottom left of each individual graph). The 10 mM $NH_4OAc$ buffer solutions (pH 6.0) contained 10 μM CB7, 2.5 μM Dapoxyl, 40 $\mu g mL^{-1}$ lysine decarboxylase, 1–9 μM L-lysine, and 41–8290 μM D-lysine.

served when the absolute concentrations of L-lysine were kept constant, but the total concentration of D/L-lysine was increased to 830 μM, corresponding to an *ee* range of 97.8–99.8%. In a final set of experiments, we prepared an extreme *ee* range of 99.78 to 99.98%, again keeping the absolute concentrations of L-lysine identical to the previous sets. The measured initial rates showed an identical linear dependence on substrate concentration ($r=0.997$, Figure 8C), which confirmed that the D form was essentially a spectator and did not affect the initial rates (see Figure 9). This is a nontrivial result, because frequently the enantiomers of the substrate do inhibit enzymatic reactions.[58–61]

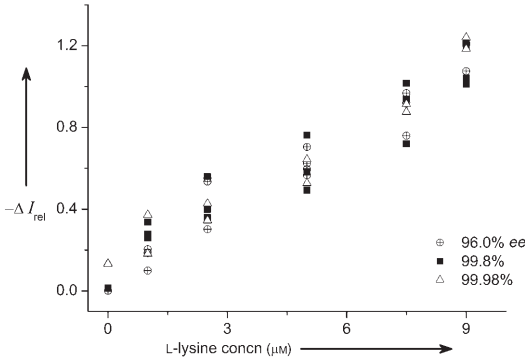



Figure 9. Combined rates of L-lysine decarboxylation as measured by a decrease in fluorescence for the three series shown in Figure 8 with maximum D-lysine enantiomeric excess as stated. The 10 mM $NH_4OAc$ buffer solutions (pH 6.0) contained 10 μM CB7, 2.5 μM Dapoxyl, 40 $\mu g mL^{-1}$ lysine decarboxylase, 1–9 μM L-lysine, and 41–8290 μM D-lysine.

The determinations are, therefore, sufficiently robust to allow an accurate determination of optical purity of the amino acid over a wide range of *ee* (64–99.98%). We constructed three calibration curves for initial rates at the different *ee* values, obtained from repetitive independent measurements (Figure 8), which could be directly employed to determine the *ee* value with high precision even in the extreme *ee* range above 99% and to allow a differentiation even between 99.86 and 99.98%. This is remarkable in comparison to conventional analytical and the more recently introduced enzymatic methods.[55,56,62] Our method is simple and quick and does not require special instrumentation. Moreover, no chemical derivatization, special sample preparation, or heating (compare GC analyses) are required. The *ee* of other amino acids (or other enzymatic substrates) could be similarly determined, although the presented case of lysine provides a particularly fortunate example due to the large substrate-product differentiation (Table 1), which will necessarily have an effect on the accessible *ee* range. Regardless of the specific implementation, however, it will always be advisable to have both pure enantiomers at hand to construct suitable reference curves for the *ee* range under investigation.

Close inspection of the trace with the neat (100%) D-amino acid sample at very high concentrations (ca. 10 mM) revealed that there was actually a very slow fluorescence decay with a non-negligible rate (arrow pointing to 100% *ee* in Figure 8C). We tentatively assign this to a trace amount of L-lysine in the commercial D-lysine sample, approximately 1 μM L enantiomer in the investigated 10 mM D-lysine solu-

tion. In fact, we can use this measurement (in comparison to the rates measured for mixtures adjusted to *ee* values around 99.95) to put a lower limit of 99.98% to the optical purity of the commercial D-lysine sample, conservatively specified as displaying an *ee* better than 99%. Unfortunately, when confronted with our result, the manufacturer was unable to confirm our finding due to the lack of available analyses with this precision.

## Conclusion

We have developed two novel applications for the recently communicated supramolecular tandem enzyme assays,[41] which couple the convenient fluorescence detection through dye displacement from macrocycles with the chemo- and enantioselectivity of enzymatic reactions. The first one involves the highly selective sensing of biomolecular analytes, either an amino acid (histidine, arginine, lysine, and tyrosine) or its corresponding decarboxylase. The detection can be transferred to a microtiter plate format with a limiting sensitivity of 2.4 nmole per well. The second application allows the determination of enantiomeric excesses of D-amino acids up to 99.98%. This establishes two instructive examples of how *unselective* recognition in supramolecular assemblies can be practically exploited.[38,63–65]

## Experimental Section

**Materials**: Fluorescence measurements were done on a Varian Cary Eclipse fluorometer ($\lambda_{exc}=336$ nm, $\lambda_{obs}=380$ nm), and the microtiter plate readings were done on a Tecan Safire instrument. The microtiter plates used were black 96-well flat-bottom plates with a nonbinding surface purchased from Corning.

Amino acids (including D-lysine) were used as received from Fluka (Seelze, Germany) and ICN Biomedicals (Aurora, Ohio). Dapoxyl was from Molecular Probes (Eugene, Oregon). Cucurbit[7]uril (CB7) was synthesized according to a literature procedure.[16,66,67] Lysine decarboxylase (partially purified, 1.6 U mg$^{-1}$), tyrosine decarboxylase (whole dried Streptococcus faecalis cells, 0.65 U mg$^{-1}$), histidine decarboxylase (crude acetone powder, 0.26 U mg$^{-1}$), and arginine decarboxylase (lyophilized powder, 2.4 U mg$^{-1}$) were used as received from Sigma-Aldrich. The buffer (10 mM) was prepared fresh on a daily basis from 100 mM stock solution and the pH was adjusted by using HCl and NaOH. Care was taken to minimize the amount of NaOH used as excessive [$Na^+$] affects complexation with CB7.[23,41] The 100 mM stock buffer was made with crystalline $NH_4OAc$ (AppliChem, Darmstadt, Germany) in ultrapure water. The reagent solutions were also prepared fresh daily in 10 mM $NH_4OAc$ buffer, pH 6.0. All experiments were conducted in this buffer.

**Cuvette-based measurements**: The initial control studies with mixtures of either three (negative control) or four (positive control) amino acids with one decarboxylase were accomplished in a fluorescence spectrophotometer with a 1 cm path length and 1 mL sample volumes. The following is a representative example of the conditions used for the tyrosine controls (Figure 3D). The enzymatic reactions were accomplished in solutions containing 2.5 μM of Dapoxyl dye, 10 μM of CB7, 50 μM of each: L-lysine, L-arginine, L-histidine, 50 μM of L-tyrosine (only in positive control), and 40 μg mL$^{-1}$ tyrosine decarboxylase.

**Microtiter plate measurements**: Solutions according to the multiparameter sensor array in Figure 4 were prepared in a 96-well microtiter plate with each well containing a total of 240 μL. The enzymatic reactions were accomplished in solutions containing: 7.5 μM Dapoxyl dye, 30 μM CB7, amino acid concentrations ranging from 1 to 1000 μM (corresponding to 0.24 to 240 nmoles per well), and either 40 μg mL$^{-1}$ of lysine or tyrosine decarboxylase, 80 μg mL$^{-1}$ of arginine decarboxylase, or 160 μg mL$^{-1}$ of histidine decarboxylase. Note two modifications compared to the cuvette-based measurements. First, to accommodate for the smaller sample volume and decreased path length of the microtiter plate the ratio of Dapoxyl and CB7 to the remaining components was increased. Second, to obtain comparable reaction rates for all enzymes on the microplate, we increased the concentrations of histidine and arginine decarboxylase, which had been found to proceed most slowly in the cuvette-based studies (Figure 3).

**Enantiomeric excess determinations**: These studies utilized a fluorescence spectrometer with a 1 cm path length and a solution volume of 1 mL. The enzymatic reactions were accomplished in solutions containing 2.5 μM of Dapoxyl dye, 10 μM of CB7, 40 μg mL$^{-1}$ lysine decarboxylase, 1–9 μM L-lysine, with either: 49–41 μM D-lysine (64–96% *ee*) or 829–821 μM D-lysine (97.8–99.8% *ee*) or 8290–8210 μM D-lysine (99.78–99.98% *ee*).

**Isothermal titration calorimetry**: Titration experiments were carried out at 30 °C in 10 mM ammonium acetate buffer, pH 6.0, on a VP-ITC calorimeter from MicroCal (USA). Each experiment consisted of 25–30 consecutive injections (5–10 μL) of guest solution (0.1–35 mM) into the microcalorimetric reaction cell containing the CB7 solution (20–50 μM). Solutions were degassed prior to titration. Heats of dilution were subtracted from each data set. The data were analyzed in Origin Pro 7.5 software (OriginLab Corporation, Northampton, MA), with the value of $n$ (number of binding sites) equal to 1, within error, consistent with a 1:1 complexation model.

**Competitive fluorescence titrations**: The fluorescence titrations were performed at ambient temperature by successive addition of known amounts of competitor to solutions containing CB7 and Dapoxyl and following the fluorescence intensity in the spectral area of largest variation. Care was taken to keep the concentration of CB7 and Dapoxyl constant in the course of the titration.

For quantitative analysis of the titrations, we define $[D]_0$, $[C]_0$, and $[M]_0$ as the total concentrations of dye, competitor (substrate or product), and macrocycle. [D], [C], and [M] are the concentrations of uncomplexed dye, uncomplexed competitor, and uncomplexed macrocycle. [M·D] and [M·C] are the concentrations of the macrocycle-dye and macrocycle-competitor complex, and $K_C$ and $K_D$ are the association constants of the competitor and dye with the macrocycle.

The fluorescence intensity ($I$) in the course of the titration can be expressed as a linear combination of the fluorescence intensity of the uncomplexed dye ($I_D$) and that of the macrocycle-dye complex ($I_{M\cdot D}$), weighted by their molar fractions according to Equation (1). $I_{M\cdot D}$ was further expressed through a 1:1 host-guest binding model[17,68,69] by the (initial) experimental fluorescence intensity ($I_0$) in the absence of competitor.

$$I = \frac{[D]}{[D]} I_D + \frac{[M \cdot D]}{[D]_0} I_{M\cdot D} \quad (1)$$

Upon appropriate substitution, one obtains Equation (2), with the concentration of uncomplexed macrocycle as a variable; the latter is defined by a cubic equation [Eq. (3)].[70]

$$I = I_D + (I_{M\cdot D} - I_D)\frac{K_D[M]}{1+K_D[M]} \quad (2)$$

$$0 = a[M]^3 + b[M]^2 + c[M] - d, \quad (3)$$

in which

$$a = K_C K_D,\ b = K_C + K_D + K_C K_D([D]_0 + [C]_0 - [M]_0)$$

$$c = K_C([C]_0 - [M]_0) + K_D([D]_0 - [M]_0) + 1, \text{ and } d = -[M]_0$$

The fitting was implemented in OriginPro 7.5 (OriginLab Corporation,

Northampton, MA), by using a subroutine to solve the cubic Equation (3) with the Newton-Raphson method. The module is available from the authors upon request.

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