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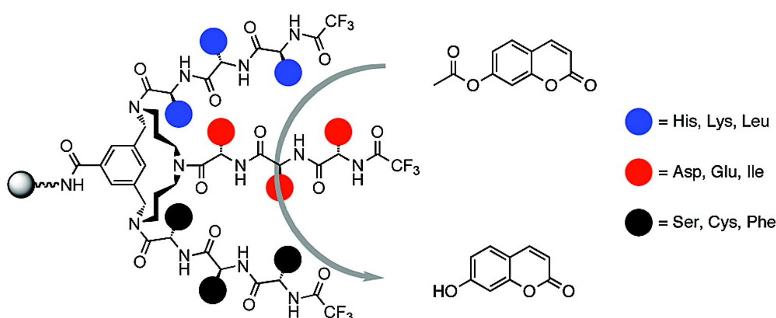

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TAC-Scaffolded Tripeptides as Artificial Hydrolytic Receptors: A Combinatorial Approach Toward Esterase Mimics

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In this report, we present the first library of tripodal synthetic receptor molecules containing three different, temporarily N-terminal protected peptide arms capable of performing hydrolytic reactions. To construct this library, the orthogonally protected triazacyclophane (TAC)-scaffold was used in the preparation of a split−mix library of 19 683 resin bound tripodal receptor molecules. For the construction of the peptide arms, three different sets of amino acids were used, each focused on one part of the catalytic triad as found in several families of hydrolytic enzymes. Therefore, in the sets of amino acids used to assemble these tripeptides, basic (containing His and Lys), nucleophilic (containing Ser and Cys), or acidic (containing Asp and Glu) amino acid residues were present. In addition, nonfunctional hydrophobic amino acid residues were introduced. Possible unfavorable electrostatic interactions of charged N-termini or their acetylation during screening were circumvented by trifluoroacetylation of the N-terminal amines. Screening was performed with a known esterase substrate, 7-acetoxycoumarin, which upon hydrolysis gave the fluorescent 7-hydroxycoumarin, leading to fluorescence of beads containing a hydrolytically active synthetic receptor. Although many synthetic receptors contain catalytic triad combinations, apparently, only a few showed hydrolytic activity. Sequence analysis of the active receptors showed that carboxylate-containing amino acids are frequently found in the acidic arm and that substrate cleavage is mediated by lysine (noncatalytic) or histidine (catalytic) residues. Kinetic analysis of resynthesized receptors showed that catalysis depended on the number of histidine residues and was not assisted by significant substrate binding.

Introduction

Artificial synthetic receptor molecules that possess catalytic properties are among the most interesting and challenging molecules to design and synthesize.[1] The combination of substrate binding and catalytic activity makes them not only interesting as catalytic species in their own respect but also, possibly, helpful in understanding enzymatic activity. Therefore, it is not surprising that enzymes have been a tremendous source of inspiration in the development of small synthetic constructs capable of performing catalytic reactions.[2] The selectivity and turn-over shown by enzymes[3] has been extremely inspiring and has posed an ever present challenge of mimicry of their behavior. Among all enzymes, serine hydrolases have received an above-average amount of attention from chemists who embarked on attempts to mimic their activity.

So far, the response to this challenge of mimicking enzymatic activity has resulted in very diverse synthetic systems ranging from complicated designed molecules with preorganized functionalities,[4] especially by Cram et al.,[5] to polymers randomly decorated with functional groups that are also present in catalytic sites of enzymes.[6] Within these approaches, peptide-based mimetics are particularly interesting[7] because their functional groups are exactly identical to their enzymatic counterparts.[8] Combinatorial approaches might be especially useful in the discovery of novel peptide based catalysts[9] or enzyme active site mimics because a broad spectrum of molecular diversity becomes available in which subtle molecular interactions can be generated beyond prediction.[9a,10] With respect to this, it should be mentioned that only two combinatorial approaches have been reported so far in which hydrolytic activity toward ester bonds could be attributed to the combination of several functionalities of the side chains of amino acids. First, the group of Reymond has shown impressive hydrolytic properties of peptidic dendrimers containing the serine protease catalytic triad aspartate-histidine-serine and serine-histidine dyads.[11] Their system showed astonishing hydrolytic activity and demonstrated unambiguously that dendrimers obtained by combinatorial approaches were 2−10 times more active than previously designed dendrimers.[12] Second, using small synthetic tweezerlike receptors, De Clercq et al. have shown that scaffolds containing two parallel tripeptides were able to cleave ester bonds, although catalysis has not been demonstrated.[13] Recently, they described an initial study on the application of an orthogonally protected tripodal synthetic receptor for the synthesis of serine protease mimics and other constructs.[14]

Encouraged by our results in the area of scaffolded peptides[15] toward selective binding of biological relevant molecules[16] and ions[17] and, more recently, as mimetics of

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Figure 1. Most important components of the active sites of cysteine or serine proteases (left) and β-lactamases and DD-peptidases (right) for hydrolysis. For clarity, the tetrahedral intermediate of the enzyme-bound substrate stabilized by the oxyanion hole is highlighted by the gray circle, and residues belonging to the enzyme are depicted in bold.

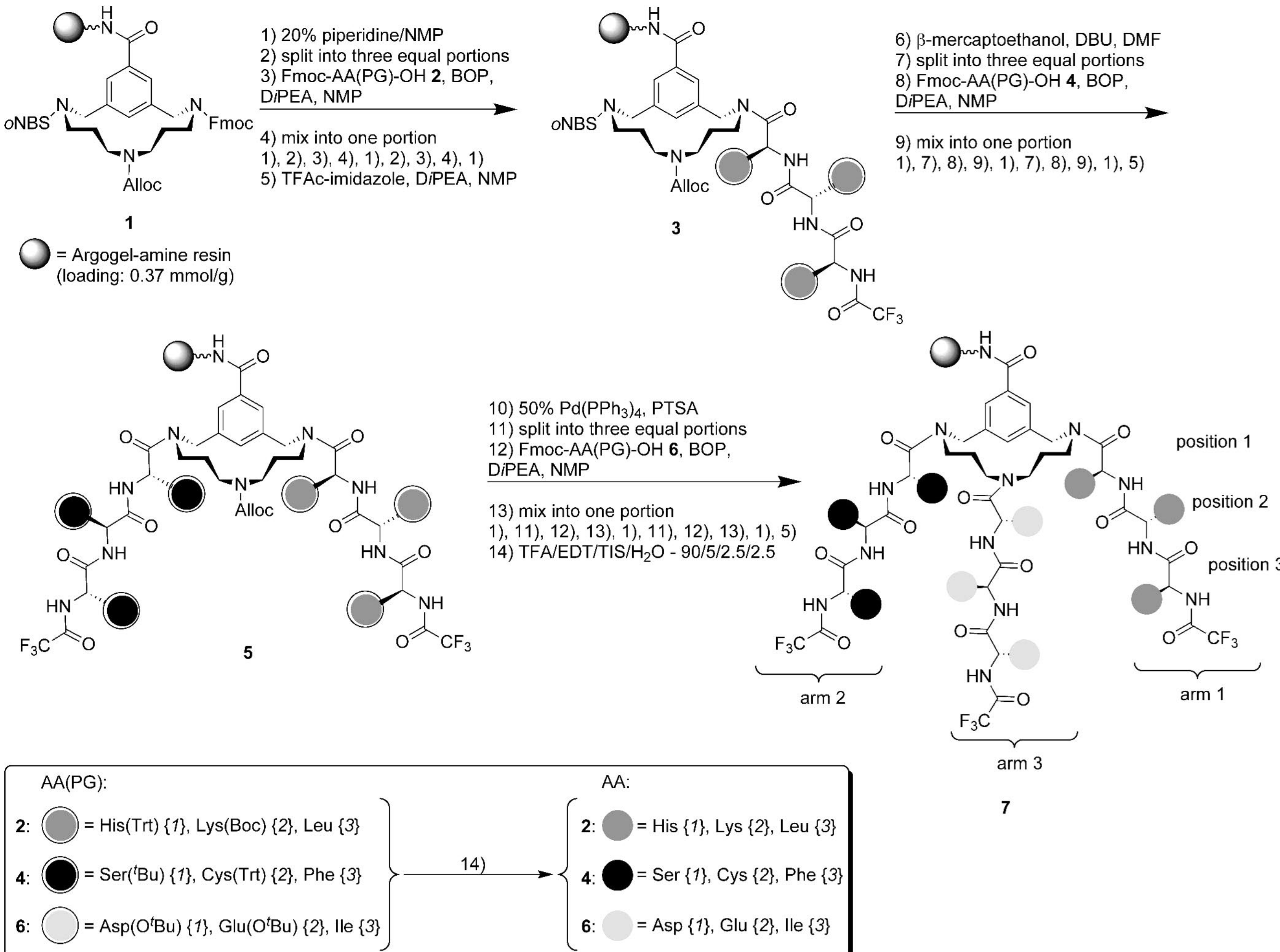
type-3 copper binding sites in proteins, especially enzymes,[18] we decided to explore the properties of TAC-based peptide receptors with respect to hydrolytic activity. These investigations might lead to peptide-based hydrolase mimics that rely on cooperation of peptide arms as a result of attachment to a suitable scaffold. In addition, we anticipated that our system with three different peptide arms attached to one scaffold could shed more light on the requirements for small systems to serve as mimics of hydrolytic enzymes. Finally, the presence of multiple functional amino acid residues that are found in numerous enzymes active sites other than hydrolytic enzymes, may lead to catalytic receptors capable of catalysis of reactions other than hydrolysis.

As far as hydrolysis by functional group enzymes is concerned, it is well-known that the activity is mainly governed by the cooperation between conformationally preorganized acidic, basic and nucleophilic residues (Asp-His-Ser/Cys in serine or cysteine proteases, and Lys-Glu-Ser in β-lactamases and DD-peptidases[19,20]) (Figure 1). On the basis of this knowledge, we decided to decorate the semiorthogonally protected triazacyclophane (TAC)-scaffold with the hydrolytically important residues that are found in the active sites of these three classes of enzymes. By selecting these residues, we created a very biased library that should allow screening for subtle arrangements of functional side chains within our synthetic receptors, possibly leading to hydrolysis. Incorporation of all these residues was deemed important in view of the relatively unknown properties of TAC-scaffolded receptors with respect to hydrolysis. The findings described in this paper can be used to create more advanced and focused libraries.

Results and Discussion

Synthesis. The aim of this study was to investigate possible hydrolytic activity of scaffolded peptides, with the ultimate goal to identify potential functional mimetics of hydrolytic enzymes. To achieve this, the three amine functionalities of the TAC-scaffold were decorated with three different tripeptide arms, using three different sets of amino acids, the chemsets. These three different chemsets enabled postscreening characterization of each of the arms in the active receptors by on-bead Edman degradation.[21] This direct identification of the amino acids present in the active receptors circumvents the use of potentially interfering tags. The amino acids used in each arm corresponded to the nucleophilic, basic or acidic part of the catalytic triad. In addition, one nonfunctional hydrophobic amino acid in each set was introduced. These nonfunctional amino acids may provide a hydrophobic binding environment for the substrate. Therefore, the used chemsets were Cys/Ser/Phe for the arm containing the nucleophile, that is, the nucleophilic set, His/Lys/Leu for the arm containing the base, that is, the basic set, and Asp/Glu/Ile for the arm containing the acid, that is, the acidic set. The resulting library was prepared by split−mix synthesis and contained theoretically 19 683 (3^9) different members of which one receptor contains only hydrophobic residues and of which 512 receptors contain only functional amino acids. A receptor in which three amino acids are specifically assigned to three positions, for instance with the serine, histidine, and aspartate catalytic triad residues attached directly to the TAC-scaffold (Scheme 1), is present in 729 (3^6) variations.

The orthogonally protected TAC-scaffold was synthesized as described before.[22] For preparation of the library by split−mix synthesis, the scaffold was attached first to the Argogel-NH_2 resin by a BOP/D*i*PEA coupling yielding **1** (Scheme 1). Removal of the Fmoc (9-fluoromethylmethoxycarbonyl) group from **1**, by piperidine in NMP, was followed by splitting of the resin into three equal portions. To each portion, one of the three amino acids from chemset **2** (Fmoc-His(Trt)-OH {*1*}, Fmoc-Lys(Boc)-OH {*2*}, Fmoc-Leu-OH {*3*}) was coupled using BOP/D*i*PEA. After coupling, the three portions of resin were mixed into one portion, and the Fmoc-group was removed using piperidine. The procedure of dividing, coupling, mixing, and deprotection procedure was repeated twice. After coupling of the last amino acid, the Fmoc-group was removed, and the liberated α-amine was protected by the base-labile trifluoroacetyl group to yield chemset **3**. This temporary N-terminal protection was introduced to avoid acylation of the α-amine during screening and prevent involvement of charged N-termini in the hydrolytic reaction. Removal of the *o*NBS (*ortho*-nitrobenzenesulfonyl) group from **3** was accomplished by β-mercaptoethanol and DBU in DMF. For the construction of the second arm, the nucleophilic chemset **4** (containing Fmoc-Ser(^{t}Bu)-OH {*1*}, Fmoc-Cys(Trt)-OH {*2*}, and Fmoc-Phe-OH {*3*}) was used in three sequential split-mix steps followed by Fmoc-group removal and trifluoroacetylation. This afforded chemset **5**. After this, the Alloc (allyloxycarbonyl) group was removed by Pd^0, and the third



Scheme 1. Synthesis of the 19 683-Membered Library in Which Each Arm of the Synthetic Receptor Corresponds to a Particular Part of Several Catalytic Triads That Are Found in Hydrolytic Enzymes (PTSA = Anilinium *p*-Toluenesulfinate)

arm was constructed using chemset **6**, consisting of Fmoc-Asp(O^{t}Bu)-OH {*1*}, Fmoc-Glu(O^{t}Bu)-OH {*2*}, and Fmoc-Ile-OH {*3*}. Removal of the N-terminal Fmoc-group was followed by trifluoroacetylation, and the side-chain protecting groups were removed using an acidic cleavage cocktail. The presence of N-terminal TFAc-protecting groups on chemset **7** was verified by resistance of the receptors toward Edman-degradation (see Supporting Information).

Screening and Sequence Analysis of Hits. For screening of the library, to assess hydrolytic activity of resin-bound receptors, the method published by Reymond et al. was used.[11a] This method involved incubation of a monolayer of receptor-containing beads with a solution of a latent fluorescent substrate in a 20 mM Bis-Tris (pH 6.0) buffer on a Petri-dish. Diffusion of the fluorescent hydrolysis product is assumed to be limited by the microenvironment created by the polymer network of the resin, resulting in a build-up of fluorescence on the active receptor containing bead. Although the screening was initially performed with several substrates,[23] the substrate that gave the best results so far was the 7-acetoxycoumarin ester. Upon hydrolysis of this substrate, the fluorescence of resulting 7-hydroxycoumarin produced a clearly identifiable blue fluorescent coloration of the bead containing the hydrolytically active receptors, which could easily be observed using a fluorescent microscope (Figure 2; excitation wavelength 340−380 nm, cutoff filter below 400 nm).

Successively increasing concentrations of substrate were applied to find supposedly less hydrolytically active synthetic receptors. Fluorescent beads were picked, transferred into a microtube, and immediately treated with a solution of acrylamide, a cysteine alkylating agent,[24] and Tesser's base,[25] subsequently. The latter reagent was needed for removal of the trifluoroacetyl groups to allow on-bead Edman-degradation.

From the Edman-degradation results, it immediately became clear that signals originating from the nucleophilic arm did not show the presence of any cysteine residues. In some degradation cycles, no signals were observed corresponding to any of the other amino acids positioned in this arm. Even more, when the library was subjected to a solution of Ellman's reagent,[26] no significant coloration of the beads or solution was seen, indicating that no cysteine thiols were present in the library. Therefore, it was assumed that the cysteine residues were oxidized to the corresponding disulfide bridges, even in cases where an odd number of cysteine residues was present on the receptor. To test this, disulfide bridges were reduced to their thiol counterparts using 1,4-dithiotreitol (DTT).[27] After extensive washing with NMP under inert atmosphere, the Ellman test indeed showed a

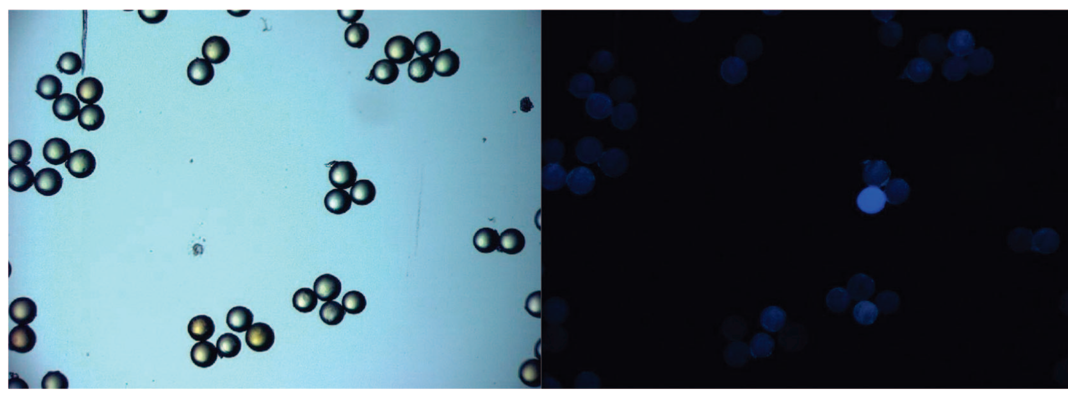
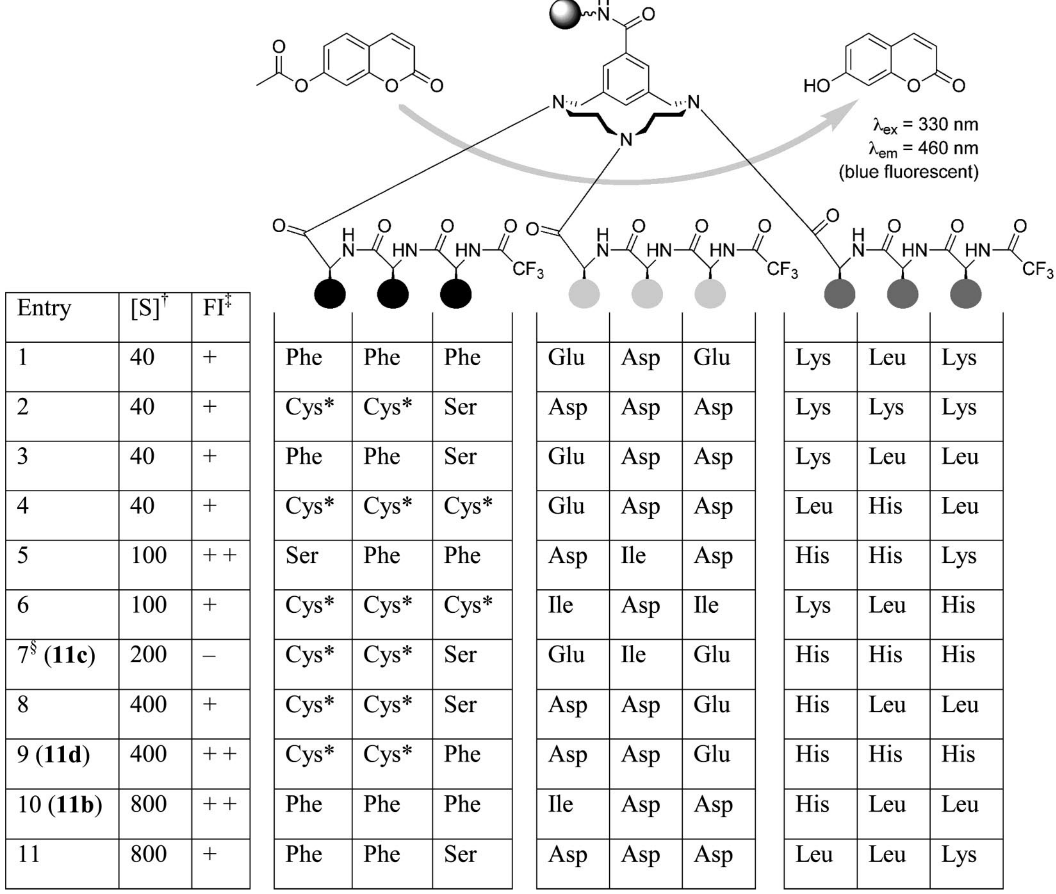


Figure 2. Clearly visible distinction between bead containing and beads lacking fluorescent hydrolyzed product (λ_{ex} = 330 nm; λ_{em} = 460 nm). Pictures obtained from beads illuminated by visible and UV (λ_{ex}: 340−380 nm) light.



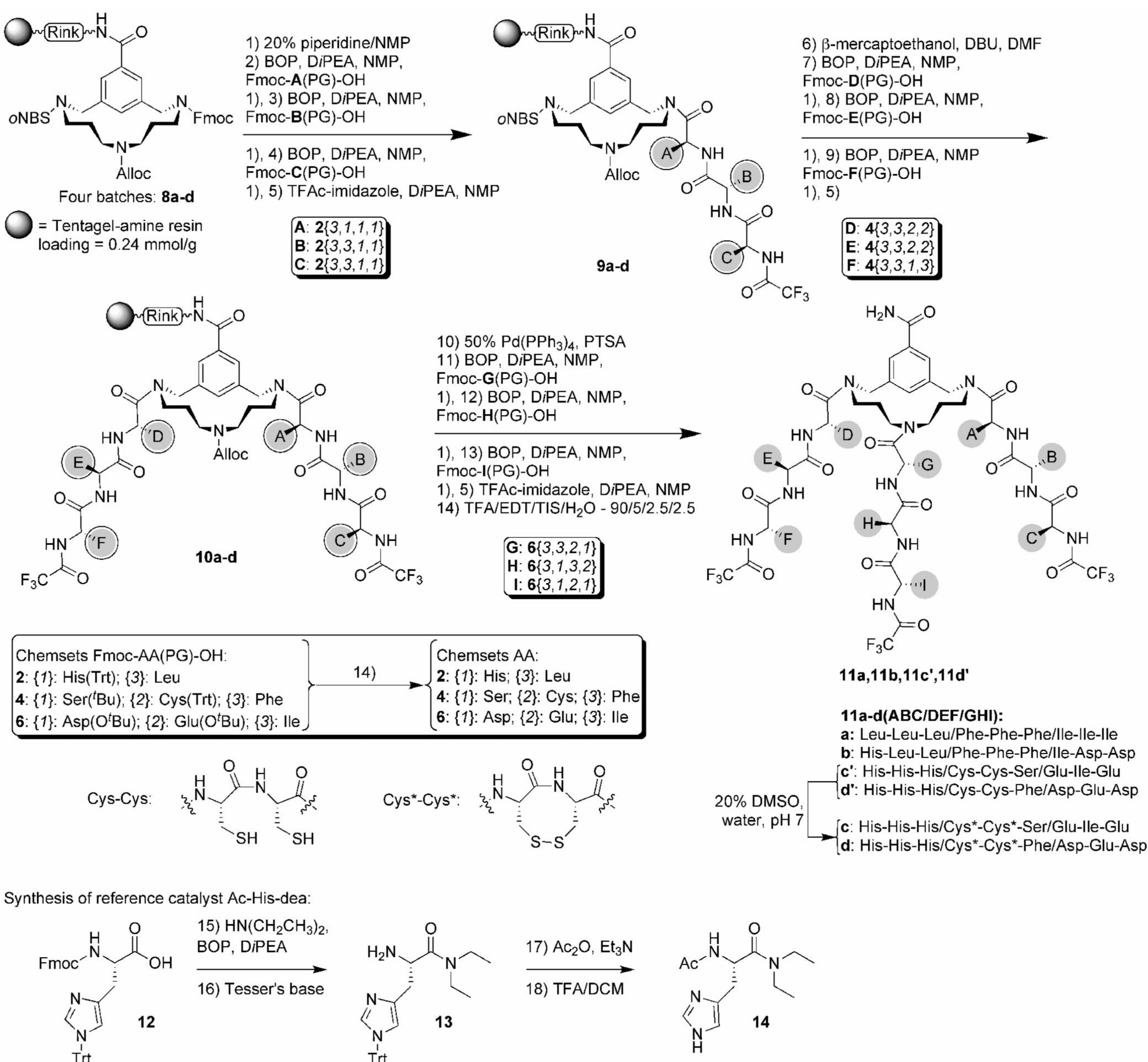
Entry	[S]†	FI‡									
1	40	+	Phe	Phe	Phe	Glu	Asp	Glu	Lys	Leu	Lys
2	40	+	Cys*	Cys*	Ser	Asp	Asp	Asp	Lys	Lys	Lys
3	40	+	Phe	Phe	Ser	Glu	Asp	Asp	Lys	Leu	Leu
4	40	+	Cys*	Cys*	Cys*	Glu	Asp	Asp	Leu	His	Leu
5	100	++	Ser	Phe	Phe	Asp	Ile	Asp	His	His	Lys
6	100	+	Cys*	Cys*	Cys*	Ile	Asp	Ile	Lys	Leu	His
7§ (**11c**)	200	–	Cys*	Cys*	Ser	Glu	Ile	Glu	His	His	His
8	400	+	Cys*	Cys*	Ser	Asp	Asp	Glu	His	Leu	Leu
9 (**11d**)	400	++	Cys*	Cys*	Phe	Asp	Asp	Glu	His	His	His
10 (**11b**)	800	++	Phe	Phe	Phe	Ile	Asp	Asp	His	Leu	Leu
11	800	+	Phe	Phe	Ser	Asp	Asp	Asp	Leu	Leu	Lys

Figure 3. Edman degradation results of found hits from screening of the library with 40−800 μM 7-acetoxycoumarin solution in 20 mM Bis-Tris buffer (pH 6.0). Cys* indicates positions of oxidized cysteine residues; bold numbers refer to resynthesized receptors (Scheme 2). † indicates that the substrate concentrations are give in μM; ‡ represents the fluorescence intesity as estimated by eye, and § respresents non-fluorescent beads as a negative control.

strong coloration of the beads and solution, indicating that cysteine residues were regenerated. Cystine and cysteine residues cannot be detected by Edman degradation,[28] and the absence of signals corresponding to acrylamide-derivatized cysteine was explained by oxidation of cysteine residues. Therefore, although signals originating from cysteine-derivatives were absent in Edman-degradation profiles, the presence of cysteine residues was assumed when none of the other two possible amino acid phenyl-thiohydantoin derivatives, that is, derived from serine or phenylalanine, showed up in the analyses. In Figure 3, these residues are indicated by "Cys*".

Some general observations can be derived from these screening results. First of all, the arm that contains residues that are known to be least important for hydrolysis mediated by serine hydrolases,[29] the middle arm with the acidic residues, shows the highest consistency with respect to the found residues by Edman degradation: the receptors preferably contain carboxylate residues. In addition, these receptors seem to prefer aspartate over glutamate. Second, despite the relatively low pH (6.0) at which the screening was performed, lysine residues were found, especially at low concentrations of screening substrate (Figure 3, entries 1−3 and 11). Although β-lactamases and DD-peptidases hydrolyze their

Scheme 2. Synthesis of Resynthesized Hydrolytic Receptors (**11a−d**) and Reference Catalyst Ac-His-dea (**14**)[a]



[a] Chemset numbering **no.**{*a*,*b*,*c*,*d*}: **no.** = number of chemset (**2**, **4**, or **6**); *a*−*d* refer to the particular amino acid of the specified chemset used for the synthesis of receptor **a**, **b**, **c**, or **d**. The circles around the shadings in **9a−d** and **10a−d** symbolize the protected nature of the side-chain functionalities; they are unprotected in **11a−d′**.

substrate using a Lys-Glu-Ser triad, receptors decorated with these triads did not show catalytic turnover. Instead, the lysine ϵ-amine reacted as a nucleophile, even though the pH was far below the pK_a of the ϵ-amine (pK_a ∼10.5),[30] resulting in aminolysis of the substrate.[31] However, increasing the substrate concentration to 100−800 μM revealed the preference of histidine over lysine residues in the basic arm of the synthetic receptors (Figure 3, entries 8−10). Third, the hits also showed that histidine residues seem to be more important for hydrolysis than serine residues. Although enzymes hydrolyze ester and amide bonds by means of direct attack of an activated serine or cysteine residue on the carbonyl carbon atom,[3] it is known that histidine residues themselves can also react as a nucleophile.[1a,2b] Even more, it has been reported that cooperation of histidine with other histidines residues[6b,c,7i,j,11d,f] or carboxylate containing residues[32] can be significantly rate enhancing. Both of these combinations are present in the hits and might have been responsible for the observed ester hydrolysis.

Resynthesis and Hydrolytic Activity. As was mentioned above, fluorescence of beads containing hits with lysine residues probably originated from aminolysis of the substrate,[31] which is a noncatalytic process. Since we were interested in catalysis, hits containing lysine residues were not considered for resynthesis.[33] With respect to this, two hits (entries 9 (**11d**) and 10 (**11b**), Figure 3), together with the negative control (entry 7 (**11c**), Figure 3) and the receptor containing nonfunctional amino acids (referred to as receptor **11a**), were resynthesized to assess their hydrolytic properties in more detail (Scheme 2). This kinetic analysis is crucial to validate the screening results and to gain a better

Table 1. Kinetic Parameters of the Resynthesized Receptors **11a**−**d** and Reference Catalyst **14**[a]

Catalyst	k_{cat} (10^{-3} min^{-1})	K_M (mM)	k_{cat}/K_M (min^{-1} mM^{-1})
receptor **11a**	-	-	-
receptor **11b**	113 ± 7	0.9 ± 0.4	0.126
receptor **11c**	332 ± 47	3.4 ± 1.5	0.098
receptor **11d**	462 ± 65	5.4 ± 1.8	0.086
Ac-His-dea **14**	471 ± 29	10.6 ± 1.3	0.044

[a] Conditions: 0.1 mM receptor **11a**−**d** or 1 mM **14**, 2.5−12.5 mM 4-nitrophenyl acetate substrate, 20 mM Bis-Tris buffer (pH 7.0) at 25 °C.

understanding of the hydrolytic properties of these molecules, which might allow further improvements of the mimics.

Synthesis of the nonresin bound receptors was performed on TentaGel S RAM resin, a resin which is decorated with an acid-labile Rink-amide linker. The same protocol used for the construction of the library was also used in these syntheses (Scheme 2), although now the products were cleaved from the resin. Receptors **11a** and **11b** yielded products of high purity[34] (see Supporting Information for HPLC-traces). Because we assumed the presence of intramolecular disulfide bonds in receptors **11c** and **11d**, cysteine-containing receptors **11c′** and **11d′** were subjected to overnight intramolecular disulfide formation using a dilute solution of the receptor in 20% DMSO in water (pH ∼7). The reaction mixture was concentrated, and pure cystine containing receptors **11c** and **11d** were obtained by preparative reversed-phase HPLC (Supporting Information).

For assessment of the hydrolytic activity of the resynthesized receptors, we studied the hydrolysis rate of 4-nitrophenyl acetate.[35] This substrate was used in view of the relatively small difference between the absorption spectrum of 7-hydroxycoumarin and 7-acetoxycoumarin, which was used for screening. During this analysis, it was observed that the rate of hydrolysis at pH 6.0 was too low to obtain any reliable kinetic data, although a significant amount of hydrolyzed product was observed after the time interval of 90 min in which the screening was performed. However, at pH 7.0 reliable kinetic data were obtained. Together with analysis of these receptors, the rates of hydrolysis without catalyst and in the presence of 4-methylimidazole (4-MeIm, a histidine side-chain mimic) and Ac-His-diethylamide (Ac-His-dea, a mimic of a histidine residue bound to a secondary amine of the TAC-scaffold) were measured (Table 1 and Scheme 2). Reference catalyst Ac-His-dea **14** was synthesized from Fmoc-His(Trt)-OH **12**. First, the diethylamide was prepared using diethylamine and BOP/D*i*PEA. The Fmoc-group was replaced with the acetyl-group by Fmoc-deprotection with base and acetylation of **13** using acetic anhy-

dride. Finally, the trityl-protecting group was removed using 5% TFA/DCM, and the final compound **14** was purified by column chromatography.

Apparently, the screening resulted in the identification of a false negative receptor **11c**, and at this stage, the origin of this false negative is not clear. The following conclusions can be derived from the results shown in Table 1. First, a correlation was present between the number of histidine residues and the rate of hydrolysis: more histidine residues resulted in higher hydrolytic activity (receptors **11c** and **11d** vs receptor **11b**). This is consistent with observations of other groups.[6b,c,7i,j,11,12] Second, the activity of the receptors is rather low: the most active receptor **11d** is only as good as Ac-His-dea, both of which are only a factor of 4.4 more active as 4-MeIm (see Supporting Information). Third, a significant difference is found between the catalytic activity of the two most active receptors (**11c** and **11d**), each containing three histidine residues in the basic arm. Apparently, variations in the amino acid sequence of the other two arms had a significant influence on the kinetic parameters of the receptor. These variations in amino acid sequence may, for example, result in a more active nucleophile, the presence of a hydrophobic substrate binding site close to the nucleophile, or even an oxyanion stabilizing moiety opposite to the attacking nucleophile. Fourth, the high values of the Michaelis constant (K_M) show that these systems do not display significant affinity for the substrate. Only receptor **11b** showed some substrate binding properties, most likely originating from the hydrophobic residues present in the receptor. Despite the higher substrate binding ability of this receptor, the activity is not comparable to the mimic of the histidine-residue in this system, that is, Ac-His-dea. This might be caused by the reduced accessibility of the imidazole ring as result of the bulkiness of surrounding amino acid residues. Poor substrate binding ability by these TAC-based receptors also followed from k_{cat}/K_M: **11b** shows the highest and **11d** the lowest value, and the low values of this quotient do not reveal the presence of a strong specific activity by any of these constructs. Lastly, even though 4-MeIm is abundantly used in the literature as a reference catalyst,[11,12] it appeared that 4-MeIm is not really a reliable mimic of histidine-based hydrolysis catalysts, although it might be used as a mimic of a histidine side-chain. When compared with Ac-His-dea, it was clear not only that the imidazole-ring is involved in catalysis but also that the backbone of the histidine residue also plays a role in the hydrolytic cleavage of the substrate.

Conclusions

Here, we describe the solid-phase preparation of a 19 683 membered library of tripodal peptide-based synthetic receptors that might act as potential functional mimetics of hydrolytic enzymes. This library was aimed at the finding of TAC-scaffolded receptors containing functional amino acids capable of hydrolyzing activated ester bonds. The availability of a sizable library allows, in principle, the evaluation of many combinations of (functional) amino acids. For this purpose, amino acids found in catalytic triads of hydrolytic enzymes were attached onto the TAC-scaffold and the N-terminal amines were temporarily protected by the trifluoroacetyl group. This protection prevented N-terminal acylation during screening, as well as possible interference of charged N-termini with catalysis. Determination of the peptide sequence of resin-bound receptors was achieved by on-bead Edman degradation after removal of the trifluoroacetyl group. Since no tags were used for decoding the selected hits, the observed activity was solely from the amino acid sequences present on the TAC-scaffold. Although screening with low concentrations of 7-acetoxycoumarin substrate gave almost exclusively hits containing the β-lactamase triad Ser-Glu/Asp-Lys, at higher concentrations hits were found containing the serine protease triad Asp-His-Ser. Unfortunately, cysteine residues in the synthetic receptors were easily oxidized and could not be made available as nucleophiles during the screening. Resynthesis and analysis of the kinetic data of a few receptors identified by the screening showed that the catalytic activity of the receptors is most likely the result of the nucleophilicity of histidinyl imidazole rings and is only assisted by low substrate binding capacity: an increasing number of histidine residues led to higher hydrolytic activity. In addition, subtle differences in the other arms had significant effect on the hydrolytic activity of the receptor. This might point to the possibilities offered by these TAC-scaffolded peptides to display catalytic activity and to tune the catalytic properties by small changes in the three peptidic arms of the receptors. Comparison of the activity of the catalytic receptors with two reference catalysts containing the imidazole ring, 4-MeIm, and Ac-His-dea showed that the activity was comparable with that of Ac-His-dea.[36] In addition, it was observed that for hydrolysis reactions, Ac-His-dea is a more reliable mimic of the histidine residue than the often-used reference compound 4-methylimidazole. On the basis of these results, more focused libraries might be prepared, containing also amino acid residues capable of binding the substrate, in addition to the catalytically active amino acid residues. With respect to this, future combinatorial approaches toward peptide-based hydrolytic receptors are best served when cysteine and lysine are replaced by other residues and when an Ac-His-amide derivative is used as a reference catalyst.

Although the catalytic activity of our synthetic receptor was low, this work shows that a multitude of diverse synthetic catalytic receptor molecule combinations can be conveniently synthesized and subjected to versatile screening procedures.[37] Even more, subtle changes in the amino acids of the arms can have pronounced effect on the catalytic activity, an observation that might encourage further research to be dedicated to the application of scaffolded peptides as catalysts. With respect to this, we envision the application of these peptide-based scaffolded receptor molecules for catalytic reactions other than hydrolysis. Finally, the comparable activity of our synthetic receptors and the reference catalyst Ac-His-dea shows that a more preorganized TAC-scaffolded systems is called for, which is under investigation.

Experimental Section

General information[17] concerning chemicals and apparatus can be found in the Supporting Information.

Standard Procedures. Generally, ~6 mL of solvent was used for each gram of resin. Fmoc-deprotection was performed twice using 20% piperidine/NMP solution, each for 8 min. After deprotection, the resin was washed with NMP (3 × 2 min) and DCM (3 × 2 min). Coupling (18 h) was performed using 4 equiv of Fmoc-AA(PG)-OH, 4 equiv of BOP, and 8 equiv of D*i*PEA in NMP in syringes placed on a shaker. Capping of amines was carried out using a standard capping reagent consisting of 0.5 M acetic anhydride, 0.125 M D*i*PEA, and 0.015 M HOBt in NMP (2 × 10 min). Standard washing was performed with NMP (3 × 2 min) and DCM (3 × 2 min). Coupling, deprotection, and capping were monitored using the Kaisertest[38] (for primary amines) or chloranil[39] test (for secondary amines).

Preparation of the Library. Prior to coupling, 1 g of Argogel-NH_2 resin (0.37 mmol/g, 65−125 mesh, average bead diameter 178 μm) was washed with 0.1 M HOBt in NMP and 15% D*i*PEA in NMP (each for 15 min) and washed with NMP and DCM. To this resin were added 715 mg (0.9 mmol, 2.5 equiv) of HO-TAC(Fmoc/Alloc/*o*NBS), 411 mg (0.9 mmol; 2.5 equiv) of BOP, and 322 μL (1.85 mmol; 5 equiv) of D*i*PEA in 10 mL NMP. The resin was shaken overnight and subsequently washed with NMP and DCM (3 × 2 min each). After capping, the resin was washed with NMP and DCM (3 × 2 min each). The resin was dried in vacuo overnight, and the loading of the resin was 0.29 mmol/g, as was determined by spectrophotometric Fmoc quantification.[40]

From half of the total amount of resin, the Fmoc-group was removed using the standard Fmoc-deprotection protocol, and the resulting N-terminal-free resin was divided into three equal portions. To each of the portions Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH, or Fmoc-Leu-OH was coupled using the standard coupling method in a volume of 3 mL of NMP. The syringes with the reagents were placed on a shaker for 18 h, after which the resin was washed using standard washing protocols and completion of the coupling was determined by means of the chloranil test. The loading of each portion of the resin was determined and corrected for the added weight (see Supporting Information). Each of the resulting resins had a loading of 0.27 mmol/g. After the resins were pooled, the Fmoc-group was removed, and the resin mixture was again divided into three equal portions. The procedure described above, involving the coupling of one of the three amino acids to each portion, was repeated twice. After this, the N-terminal Fmoc-group was removed from the resulting scaffolded tripeptide, and the α-amine group was protected with the trifluoroacetyl group using 248 μL of D*i*PEA (20 equiv, 0.713 mmol) and 163 μL of 1-(trifluoroacetyl)imidazole (20 equiv) in NMP. The reaction was allowed to proceed for 18 h under gentle shaking. The resin was washed with NMP and DCM (each 3 × 2 min), and completion of protection of the first arm was shown by a negative Kaiser test.

Prior to the removal of the *o*NBS-group, the resin was thoroughly washed with DMF to remove all DCM. After this, the *o*NBS-group of the scaffold was removed using 1.43 mL of 0.5 M 2-mercaptoethanol in DMF (5 equiv, 0.713 mmol) and 53 μL of DBU (2.5 equiv, 0.356 mmol) (2 × 30 min).[41] After deprotection, the resin was washed using DMF (3 × 2 min) and DCM (3 × 2 min). A positive chloranil test showed the presence of a secondary amine. The resin was divided into three equal portions; to each of which, one of the amino acids of Fmoc-Cys(Trt)-OH, Fmoc-Ser(^{t}Bu)-OH or Fmoc-Phe-OH was attached using the standard coupling procedure. The synthesis of this, so-called "nucleophilic", arm was completed as described for the first, "basic" arm.

Subsequently, the Alloc-group was removed using 50% $Pd(PPh_3)_4$ (41 mg) in the presence of 20 equiv of anilinium *p*-toluenesulfinate as a scavenger (378 mg) in NMP. The reaction was carried out for 18 h under a gentle stream of argon and liberation of the amine was apparent from the chloranil test. The construction of the third, "acidic" arm was carried out using the same procedure as for the other two arms, using the amino acids Fmoc-Asp(O^{t}Bu)-OH, Fmoc-Glu(O^{t}Bu)-OH, and Fmoc-Ile-OH. After Fmoc-deprotection of the tripeptide and trifluoroacetylation of the N-terminal amine, the fully protected library was obtained. Deprotection of the side-chains was carried out using an acidic cleavage cocktail of TFA/EDT/TIS/H_2O, 90/5/2.5/2.5 (%), for 3 h. The resin was thoroughly washed with NMP and DCM, until no EDT could be smelled, and was stored under argon.

Resynthesis of the Hits from Screening, That Is, Receptors 11a−d. Resynthesis of the receptors was carried out analogously to the procedure used for the construction of the library. After the deprotection and cleavage, the receptors containing the two cysteine residues (**11c′** and **11d′**) were subjected to disulfide bridge formation. This could be achieved by overnight reaction of 1 mM solutions of the receptors **11c′** and **11d′** in 20% DMSO/water at pH ~7. After this, the reaction mixture was condensed in vacuum at 40 °C and the crude mixture was purified by preparative HPLC. The products were analyzed by MS, and the combined fractions containing the products were lyophilized. MS-analysis of resynthesized receptors: receptor **11a** calcd $[M + H]^+ = 1684.85$, found $[M + H]^+ = 1685.35$; receptor **11b** calcd $[M + H]^+ = 1712.71$, found $[M + H]^+ = 1711.95$; receptor **11c** calcd $[M + H]^+ = 1638.52$, found $[M + H]^+ = 1638.62$; receptor **11d** calcd $[M + H]^+ = 1686.49$, found $[M + H]^+ = 1686.36$. HPLC traces of **11a–11d** can be found in the Supporting Information.

7-Acetoxycoumarin Ester. The synthesis of 7-acetoxycoumarin ester was carried out according to the literature.[42]

Screening. Prior to screening, the library was washed with 25% D*i*PEA (1 × 10 min) under nitrogen to remove the TFA-salts that were left after deprotection, followed by NMP (3 × 2 min, 6 mL each time), DCM (2 × 2 min, 6 mL each time), MeCN (2 × 2 min, 6 mL each time), and water (2 × 2 min, 6 mL each time). Amino acid side-chain amine, carboxylic acid, and imidazole functionalities were protonated or deprotonated, respectively, by incubation with 20 mM Bis-Tris (pH 6.0) buffer overnight. The buffer was removed; the resin was quickly rinsed with MeCN and poured into a Petri dish using DCM. This created, upon evaporation of the DCM, a perfect monolayer, necessary for screening. The library was treated with a small amount of

MeCN to remove residual DCM. After evaporation of all MeCN a substrate solution in the buffer, a stock solution of the substrate in MeCN was diluted a 1000-fold with the buffer to the appropriate concentration, was applied and incubated with the resin on the Petri dish for 90 min. Blue fluorescent beads, indicating hydrolysis by synthetic receptors, were visualized under a fluorescent microscope. Fluorescent beads were picked and processed further in micro tubes (100 μL). Prior to subsequent screenings, the library was washed in a syringe with frit using MeCN, NMP, and MeCN. Beads were again poured into the Petri dish according to the procedure mentioned above.

Postscreening Alkylation of Cysteine Residues and Removal of the Trifluoroacetyl Group. This was performed to prepare the library for on-bead Edman sequencing. Cysteine residues were alkylated by treatment of the bead with a 2 M solution of acrylamide in 0.3 M Tris buffer (pH 8.3) for 2 h in the dark at room temperature under argon. After alkylation, excess of reagents was removed by washing with water (3 times), ether (once), DCM (once), and MeOH (twice). Removal of N-terminal trifluoroacetyl-groups was performed by treatment with Tesser's base[25] (overnight) and subsequent washing with water (twice). After this, beads were subjected to on-bead Edman sequencing (Supporting Information).

1,4-Dithiotreitol (DTT) Reduction of Cysteine Residues. A solution of 17.1 mg (111 μmol, ~3 equiv/Cys) DTT was dissolved in a minimum amount of 0.3 M Tris-buffer (pH 8.3). This was added to the library and allowed to react for 30 min. During disulfide reduction, the beads turned slightly brown. The DTT solution was removed by filtration, and the resin was washed with 1 mL of aqueous 0.1 M EDTA solution in 2 mL of NMP (2 × 2 min); this resulted in complete decoloration of the beads. After this, the beads were extensively washed with NMP under inert atmosphere.

Ellman's Test. The dry resin was swelled in DCM and washed twice with MeCN, followed by a quick rinse with water. A solution of 3 mM EDTA in a 200 mM NaOAc (pH 8.0) buffer was added, and the mixture was allowed to react for 5 min. The solution was removed by suction, and the beads were treated with a 20 mM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) solution in 50 mM phosphate buffer (pH 7.0) under a gentle stream of nitrogen. DTT treated resin showed an immediate color change to yellow (caused by the formation of the thiolate anion) upon treatment with Ellman's reagent. Resin not treated with DTT did not undergo this color change. The resulting mixed cysteine-Ellman reagent disulfides could again be reduced by DTT, resulting in a strongly yellow colored solution, indicating regeneration of cysteine residues.

Synthesis of Ac-His-diethylamide. Fmoc-His(Trt)-OH (1 mmol, 620 mg) was dissolved in DCM, and 1 equiv of BOP (442 mg) and 2 equiv of D*i*PEA (348 μL) were added. To this solution, a solution of 1 equiv of diethylamine (104 μL) in 10 mL of DCM was added slowly. After completion of the reaction (~2 h), the mixture was concentrated under reduced pressure.

^{1}H NMR ($CDCl_3$): δ 1.00−1.05 + 1.14−1.26 (2 × 3H, dt, 2 × CH_2CH_3), 2.80−3.00 (2H, dq, $C^{\beta}H_2$), 3.16−3.23 + 3.43−3.47 (4H, dm, NCH_2), 4.08−4.27 (3H, dm, Fmoc-$CHCH_2$), 4.87−4.90 (1H, q, $C^{\alpha}H$), 5.75−5.78 (1H, d, C(O)N*H*), 6.61 (1H, s, $C^{\delta}H$), 7.07−7.74 (24H, m, Trt-C*H* + Fmoc-$C^{Ar}H$).

To the crude mixture, 25 mL of Tesser's base (1,4-dioxane/MeOH/4N NaOH = 14/5/1)[25] was added, and the solution was stirred for 30 min at room temperature. The basic mixture was acidified with an excess of 1 N $KHSO_4$, and organic material was removed by extraction with diethylether. After this, the aqueous phase was adjusted to basic pH (~12) by addition of 4 N NaOH, and H-His(Trt)-diethylamide was extracted with EtOAc. This organic phase was concentrated in vacuo. The product was dissolved in 30 mL of DCM and 1.5 equiv of Et_3N (418 μL), together with 1.5 equiv Ac_2O (284 μL), were added. The mixture was stirred overnight and concentrated under reduced pressure. The product was dissolved 1 N $KHSO_4$ and extracted from the water phase by repeated extractions with EtOAc. The organic phase was washed with brine, dried over Na_2SO_4, and concentrated in vacuo.

^{1}H NMR ($CDCl_3$): δ 1.00−1.04 + 1.16−1.21 (2 × 3H, dt, 2 × CH_2CH_3), 1.90 (3H, s, Ac−CH_3), 2.77−2.98 (2H, dq, $C^{\beta}H_2$), 3.13−3.30 + 3.40−3.47 (4H, dm and m, NCH_2), 5.10−5.13 (1H, q, $C^{\alpha}H$), 6.58 (1H, d, $C^{\delta}H$), 6.63−6.65 (1H, d, C(O)N*H*), 7.08−7.15 (6H, m, Trt-$C^{2,6}H$), 7.26−7.29 (1H, d, $C^{\epsilon}H$), 7.29−7.34 (12H, m, Trt-$C^{3,4,5}H$).

Removal of the trityl-protecting group was performed by dissolving the product in 5% TFA/DCM. After concentration, the crude material was dissolved in 1 N $KHSO_4$ and washed with DCM. To the aqueous solution of the product, $NaHCO_3$ (s) was added until pH ~9 (pH paper), and the product was extracted by repeated washings with EtOAc. A final purification by column chromatography (10−20% MeOH/DCM gradient) afforded the pure product Ac-His-diethylamide as a colorless oil (overall yield: 9.5 mg, 4%).

R_f (20% MeOH/DCM): 0.56. Supporting Information-MS: m/z 253.46 (calcd 253.17 $[M + H]^+$); 275.59 (calcd $[M + Na]^+$ 275.15); 527.35 (calcd $[2M + Na]^+$ 527.32). ^{1}H NMR ($CDCl_3$): δ 1.07−1.16 (6H, dt, J = 6.9 and 7.2 Hz, 2 × CH_2CH_3), 1.99 (3H, s, Ac−CH_3), 2.93−3.09 (2H, dq, J = 6.3 and 8.5 Hz, $C^{\beta}H_2$), 3.16−3.36 + 3.45−3.54 (4H, dm, NCH_2), 5.06−5.13 (1H, q, J = 6.6 and 8.3 Hz, $C^{\alpha}H$), 5.8 (1H, br, Im-N*H*), 6.83 (1H, s, $C^{\delta}H$), 6.94−6.97 (1H, d, J = 8.3 Hz, C(O)N*H*), 7.56 (1H, s, $C^{\epsilon}H$). ^{13}C NMR (APT, DMSO-d_6): δ 12.8−14.2 (d, CH_2CH_3), 22.3 (C(O)CH_3), 29.8 (C^{β}), 41.1 (CH_2CH_3), 48.3 (C^{α}), 117.1 (C^{δ}), 127.7 (C^{γ}), 134.4 (C^{ϵ}), 168.6 (*C*(O)N), 170.1 (*C*(O)NH).

Hydrolysis Studies. To analyze the hydrolytic properties of the free receptors under identical conditions, hydrolysis was performed in 96-well plates. For this, the resynthesized receptors or reference catalysts were dissolved in DMSO to a concentration of 0.1 and 1 mM, respectively, and 4-nitrophenyl acetate was dissolved in a mixture of DMSO/buffer, 2/3 (v/v), to a concentration of 25 mM. For the buffer, 20 mM Bis-Tris (pH 6.0 and 7.0) was used. All measurements were carried out with a total volume of 50 μL (5 μL "catalyst" + 5−25 μL substrate solution + 40−20 μL buffer). The reaction was followed for 2 h, and initial rates were calculated using the steepest increment over five

measurements of the first ten minutes. This steepest increment (mOD/min) was given by the software used (Full Mode-KC4 (version 3.4 (rev 21)) software (BioTek instruments); data point resolution was set at 2 nm). This rate v (mOD/min) was converted into the rate in μM/min using the appropriate calibration curves made from solutions of 4-nitrophenol in 50 μL buffer/DMSO mixtures. All hydrolytic reactions were performed in duplicate. After subtraction of the background hydrolysis, the obtained V_{net}(μM/min)/[S](mM) values were processed using GraphPad Prism 4 (see Supporting Information). V_{max} and K_M were calculated using the nonlinear fit model of the program, which relies on the formula $V_{max} = V_{net}$*[pNAc])/(K_M+[S]). The turnover (k_{cat}) number was calculated by V_{max}/[cat]. See Supporting Information for V_{net}/[S] curves and for more information.

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Supporting Information Available. General information on chemicals and apparatus. Edman degradation profiles, HPLC traces of the resynthesized receptors, and detailed information on the kinetic analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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(34) It should be mentioned that the total number of synthetic steps for the synthesis of the library and the resynthesized receptors is 27. The isolated yield of receptors **11a** and **11b** was 18−22%, which corresponds to an average yield of 94% per step; yields of **11c** and **11d** were lower because of the extra steps needed in the synthesis.

(35) The use of two different substrates, one to meet screening conditions and another one to meet the conditions needed for the determination of catalytic parameters, is not uncommon. For instance, see: Schmuck, C.; Dudaczek, J. *Org. Lett.* **2007**, *9*, 5389–5392.

(36) It should be noted that peptides cleaved from the resin were isolated as TFA-salts after lyophilization. Receptors **11b** (one histidine residue), **11c**, and **11d** (each containing three histidine residues), assuming the presence of one TFA counterion per histidine residue, therefore have molecular weights of 1826.73, 1980.62, and 2028.62, respectively. Using these values, the molarity of catalyst in the hydrolysis studies becomes then 93.7, 82.7, and 83.1 mM, respectively. These values were used in the calculation of the k_{cat} (100 mM is used for receptor **11a**).

(37) Other substrates might be screened by this library. The addition of transition metals also might facilitate reactivity. For instance, see: (a) Berkessel, A.; Hérault, D. A. *Angew. Chem., Int. Ed.* **1999**, *38*, 102–105. (b) Lavastre, O.; Bonnette, F.; Gallard, L. *Curr. Opin. Chem. Biol.* **2004**, *8*, 311–318.

(38) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595–598.

(39) Vojkovsky, T. *Pept. Res.* **1995**, *8*, 236–237.

(40) Using a slightly modified procedure of Chan, W. C.; White, P. D. *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*; Oxford University Press: New York, 2000; pp 62−63. See Supporting Information.

(41) For instance, see: Fukuyama, T.; Jow, C.-K.; Cheung, M. *Tetrahedron Lett.* **1995**, *36*, 6373–6374.

(42) A slightly modified procedure was used from Ren, X.; Chen, X.; Peng, K.; Xie, X.; Xia, Y.; Pan, X. *Tetrahedron Asymmetry* **2002**, *13*, 1799–1804.

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