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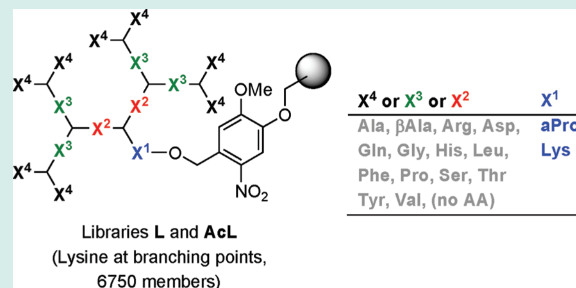
# Combinatorial Discovery of Peptide Dendrimer Enzyme Models Hydrolyzing Isobutyryl Fluorescein

Noélie Maillard, Rasomoy Biswas, Tamis Darbre, and Jean-Louis Reymond*

Department of Chemistry and Biochemistry, University of Berne, Freiestrasse 3, CH-3012, Berne, Switzerland

Supporting Information

**ABSTRACT:** Two 6750-membered one-bead-one-compound peptide dendrimer combinatorial libraries **L** $(X^4)_8(LysX^3)_4(LysX^2)_2LysX^1$ ($X^{1-4}$ = 14 different amino acids or deletion, *Lys* = branching lysine residue) and **AcL** (with N-terminal acetylation) were prepared by split-and-mix solid phase peptide synthesis. Screening toward fluorogenic substrates for esterase and aldolase activities using the *in silica* off-bead assay (N. Maillard et al., *J. Comb. Chem.* **2009**, *11*, 667−675) and bead decoding by amino acid analysis revealed histidine containing sequences active against fluorescein diacetate. Isobutyryl fluorescein, a related hydrophobic fluorogenic substrate, was preferentially hydrolyzed by dendrimers from library **AcL** containing hydrophobic residues such as **AcH3** $(AcHis)_8(LysLeu)_4(LysVal)_2LysLysOH$, compared to simple oligohistidine peptides as reference catalysts. Polycationic dendrimers from library **L** with multiple free N-termini such as **H8** $(His)_8(Lys\beta Ala)_4(LysThr)_2LysaProNH_2$ (aPro = (2*S*,4*S*)-4-aminoproline) showed stronger reactivity toward 8-acetoxypyrene-1,3,6-trisulfonate with partial acylation of N-termini. These experiments highlight the role of noncatalytic amino acids to determine substrate selectivity in peptide dendrimer esterase models.




**KEYWORDS:** combinatorial libraries, peptide dendrimer, hydrolysis, isobutyryl fluorescein, enzyme models

## INTRODUCTION

Dendrimers are regularly branched tree-like synthetic macromolecules displaying a range of interesting properties.[1] Most dendrimers consist of predefined organic cores to which variable end groups are attached for function.[2] Recently we reported that peptide dendrimers can be obtained by solid-phase peptide synthesis alternating branching diamino acids and standard α-amino acids in the sequence.[3] Peptide dendrimers resemble proteins in their composition and adopt a globular shape because of their topology rather than by folding. In approaches similar to those reported by others for libraries of linear peptides,[4,5] our screening of one-bead-one-compound (OBOC) combinatorial libraries[6] identified peptide dendrimers with various functions such as esterase[7] and aldolase[8] catalysis and selective binding to metal ions,[9] cofactors,[10] and proteins.[11] In the case of enzyme model studies for esterase activities, our experiments best succeeded when using charged fluorogenic substrates such as 8-acetoxypyrene-1,3,6-trisulfonate **4** (Scheme 1). On the other hand screening with hydrophobic, non charged substrates generally failed to identify significant catalysis in any of the libraries.

The peptide dendrimer combinatorial libraries screened for esterase catalysis were based on sequence designs in which each amino acid appeared only twice in the synthesis in two successive branches. This design produced library sizes manageable by SPPS, which is limited by the number of solid support beads in the experiment, and enabled decoding by the inexpensive and efficient amino acid analysis (AAA) of the beads. However, with this approach only a small fraction of the theoretically possible peptide dendrimer sequence space was actually surveyed. Indeed in a typical third generation peptide dendrimer library with eight variable amino acid positions with sequence $(X^8X^7)_8(BX^6X^5)_4$-$(BX^4X^3)_2BX^2X^1$ ($X^{1-8}$ = variable amino acid, *B* = branching diamino acid), a choice of 15 amino acids implied that over 2 billion possible sequences were possible, while we used only four different amino acids per variable position and thus prepared 65,536 sequences, corresponding to only 0.0026% of the theoretically possible sequence space. This left open the possibility that the difficulty to identify esterase catalysts toward hydrophobic substrates was caused by incomplete screening of sequence space.

In our efforts to realize a more thorough coverage of peptide dendrimer sequence space, we herein report the screening of the 6750-membered combinatorial libraries **L** $(X^4)_8(LysX^3)_4$-$(LysX^2)_2LysX^1$ ($X^{1-4}$ = variable amino acid, *Lys* = branching lysine residue) and **AcL** (with N-terminal acetylation, Figure 1) with more extensive survey of sequence space for three of its four variable positions.The library was screened for conversion of various fluorogenic substrates for aldolase and esterase activities using our recently reported off-bead catalysis assay, which allows to assay the libraries in solution on the surface of the silica gel

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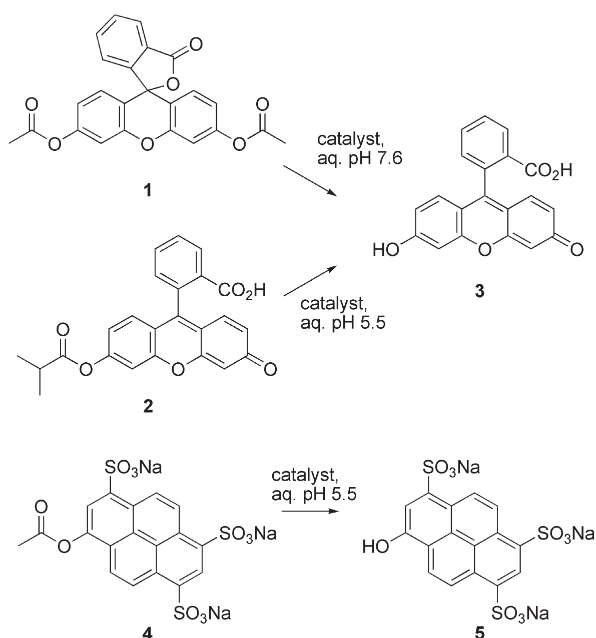
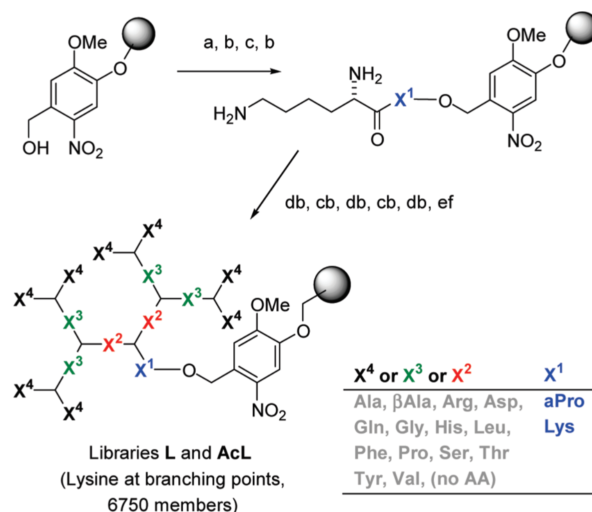
**Scheme 1. Peptide Dendrimer Catalyzed Fluorogenic Ester Hydrolysis Reactions**



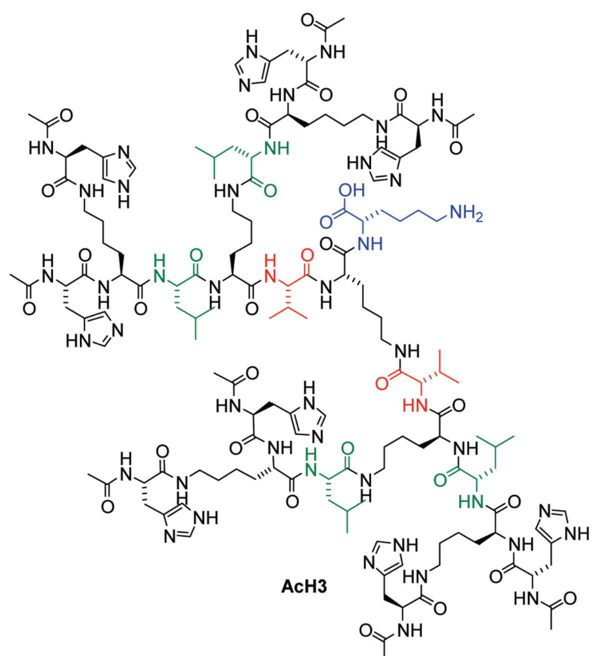




**Figure 1.** Synthesis of third generation peptide dendrimer combinatorial libraries AcL and L. Conditions: (a) MSNT (1-(2-mesitylenesulfonyl)-3-nitro-1*H*-1,2,4-triazole), Fmoc$X^1$OH, 1-methylimidazole, $CH_2Cl_2$, 1 h, (2×); (b) piperidine 20% in DMF (2 × 10 min); (c) FmocLys(Fmoc)OH, PyBop, iPrNEt$_2$, NMP; (d) split in 15 portions, then Fmoc$X^i$OH, PyBop, iPrNEt$_2$, NMP; (e) for AcL: $Ac_2O/CH_2Cl_2$ (1:1), 1 h; (f) TFA/TIS/$H_2O$ (94:5:1), 4 h. aPro = (2*S*,4*S*)-4-aminoproline, introduced with (*N*-Boc-*trans*-4-*N*-Fmoc-amino-L-proline).

plate, a method which is less prone to bead-surface artifacts than simple on-bead activity assays.[12] The screening led to the identification of several sequences catalyzing the hydrolysis of fluorescein diacetate **1**. A comparative kinetic study with isobutyryl fluorescein **2** as a model hydrophobic substrate and 8-acetoxypyrene-1,3,6-trisulfonate **4** as a typical polyanionic substrate reveals unprecedented aspects of structure-activity relationships in peptide dendrimer enzyme models, in particular the role of noncatalytic amino acids to determine substrate selectivity.

## RESULTS AND DISCUSSION

**Library Design.** AAA is applicable for decoding SPPS synthesis beads because the amount of peptide per bead is sufficient for the sensitivity of AAA, which requires at least 90 pmols. While our previous examples of AAA decodable libraries were limited to cases where each amino acid is only used in two of the variable positions during library synthesis, the method is theoretically suitable for decoding an exhaustive combinatorial library of third generation peptide dendrimers if each branch bears only a single variable amino acid position, as shown in Table 1.

Taking into account the possible difficulties in uniquely resolving the copy number above 10 copies by AAA, we set out to explore this exhaustive library design in a focused example considering the variable positions $X^2$, $X^3$, and $X^4$ (Figure 1). The core position $X^1$ would be limited to 4-aminoproline or lysine only to selectively explore single-site enamine-type organocatalytic aldolase dendrimers, a type of activity previously identified in peptides[13] and peptide dendrimers.[8] Fourteen amino acids were included for the variable positions including aromatic (phenylalanine and tyrosine), hydrophobic (leucine, proline, and valine), small and polar (alanine, glutamine, glycine, serine, and threonine), charged and catalytic residues (arginine, aspartate, histidine). $\beta$-alanine and a deletion (which can be identified by AAA) were also included to allow size variation in the dendrimer. We excluded tryptophan (not detectable by AAA), glutamate and asparagine (indistinguishable in AAA from glutamine respectively aspartate), isoleucine (too similar to leucine), lysine (already used as branching unit), and sulfur containing amino acids.

**Library Synthesis and Screening.** The library was prepared by split-and-mix Fmoc-SPPS on a 1.5 g batch of hydroxymethyl-photolinker NovaSyn TG resin (0.24 mmol/g, bead diameter 130 $\mu$m, ~8 × $10^5$ bead/g, 200−300 pmol/bead). After the final Fmoc deprotection, library **L** was either used directly, thus

**Table 1. Amino Acid Copy Numbers from AAA and Position Attribution in a 3rd Generation Peptide Dendrimer Library $(X^4)_8(LysX^3)_4(LysX^2)_2LysX^1$**

| copy number from AAA | positions occupied |
|---|---|
| 1 | $X^1$ |
| 2 | $2\ X^2$ |
| 3 | $2\ X^2 + X^1$ |
| 4 | $4\ X^3$ |
| 5 | $4\ X^3 + X^1$ |
| 6 | $4\ X^3 + 2\ X^2$ |
| 7 | $4\ X^3 + 2\ X^2 + X^1$ |
| 8 | $8\ X^4$ |
| 9 | $8\ X^4 + X^1$ |
| 10 | $8\ X^4 + 2\ X^2$ |
| 11 | $8\ X^4 + 2\ X^2 + X^1$ |
| 12 | $8\ X^4 + 4\ X^3$ |
| 13 | $8\ X^4 + 4\ X^3 + X^1$ |
| 14 | $8\ X^4 + 4\ X^3 + 2\ X^2$ |
| 15 | $8\ X^4 + 4\ X^3 + 2\ X^2 + X^1$ |

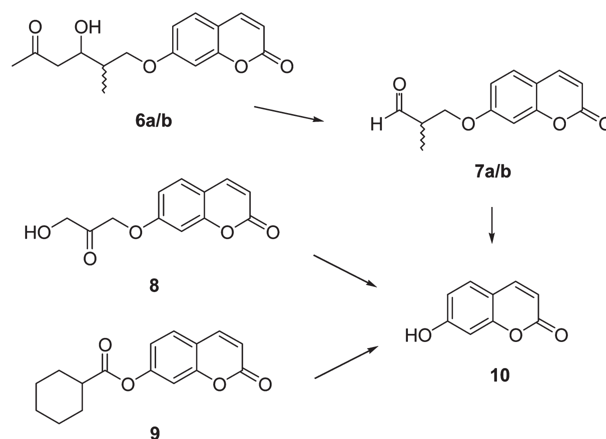
featuring multiple free N-termini with possible aldolase type catalytic properties, or acetylated to provide library **AcL** in which only the lysine or aminoproline residue at position $X^1$ displayed an enamine reactive amino acid. The library quality was checked by submitting 72 beads to AAA. A total of 56 beads (78%) returned a readable sequence, showing the expected even distribution of amino acids (Supporting Information, Table S1). The remaining beads contained 10 incomplete sequences (14%) and 6 empty beads (8%) reflecting the yield of library synthesis.

Screening was performed by off-bead screening following our recently reported protocol.[12] In this assay the library beads are partially photolyzed in the absence of solvent, and subsequently placed on the surface of a silicagel plate freshly impregnated with a solution of a fluorogenic substrate for the reaction of interest. The photolyzed portion of the dendrimer dissolves and diffuses into the surrounding wet silicagel. Synthesis beads carrying an active sequence are detected by the formation of a fluorescent halo indicating product formation. The beads are then retrieved with a pipet, washed, and subjected to decoding by AAA of the nonphotolyzed portion of the dendrimer which has remained attached to the bead. Decoding requires only total hydrolysis under acidic conditions, amino acid derivatization, and a single HPLC analysis per bead to give the amino acid composition. This method is much simpler than Edman sequencing, does not require a free N-terminus, and can even be adapted to the analysis of linear and cyclic peptide libraries.[14]

The procedure was applied by photolyzing 10 mg portions of libraries **L** and **AcL** in the absence of solvent and applying the beads to silica gel plates freshly impregnated with aqueous buffered solutions of the fluorogenic substrates at 200 $\mu$M in the case of the water-soluble substrates **6** and **8**. For the water-insoluble substrate **1** and **9** the silicagel plates were first impregnated with a 100 $\mu$M solution of the fluorogenic substrate in $CH_2Cl_2$, dried, and impregnated with aqueous buffer prior to applying the library beads. Substrates **6a**/**b**, **8**, and **9** release hydroxycoumarin by retroaldol reaction (**6a**/**b**), enediol formation, and tautomerization (**8**) or ester hydrolysis (**9**).

The tests with the aldol-type fluorogenic substrates **6**, **8**, and **9** were all negative, suggesting that enamine-type reactivity, which

**Scheme 2. Reactions of Fluorogenic Substrates That Gave No Hits in the off-Bead Assays with Libraries L and AcL**



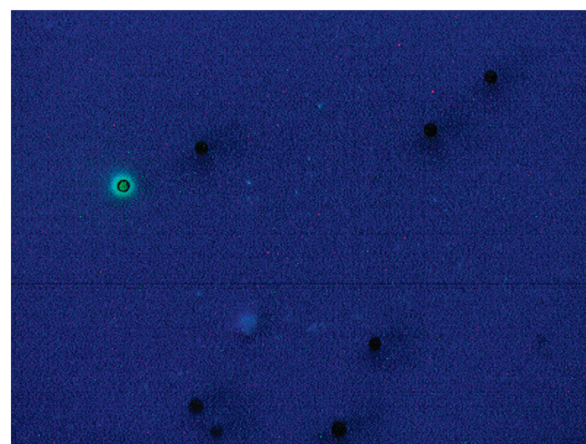



**Figure 2.** Fluorescent active halo from an off-bead screening with fluorescein diacetate **1**. The photolyzed beads (here **AcL**) were poured on an impregnated TLC with PB buffer 60 mM pH 7.6 and 100 $\mu$M **1**. The picture is taken under illumination with a 366 nm TLC-UV lamp.

was expected to involve with either the primary or the secondary α-amino group of the C-terminal amino acid at the core position $\mathbf{X^1}$ in the case of library **AcL** or the multiple N-terminal amino group in the case of library **L**, did not occur at a detectable level in this assay (Scheme 2). While the hydrophobic esterase substrate **9** was also unreactive, active beads were observed in the case of the esterase substrate **1** for both libraries **L** and **AcL** (Figure 2). In each assay with **1** a fluorescent halo appeared after 24 h at room temperature (RT) around 7−9 beads (0.1% of approximately 8,000 beads). These active beads as well as a selection of control beads without halo were transferred to vials and subjected to AAA. From 50 beads picked in the assays with **AcL** and **L**, 37 returned a readable sequence (74%, Table 2), 8 contained incomplete sequences (16%), and 5 were empty (10%). The analysis of the 60 non-hits provided 43 readable sequences (72%) (Supporting Information, Table S2), 6 incomplete sequences (10%), and 11 empty beads (18%). It should be noted that approximately 5−10% of the positive hit beads were aggregated in groups of 2−5 beads, in which case the beads were not analyzed.

**Table 2. Sequences for 37 Positive Hits in the off-Bead Assay with Fluorescein Diacetate 1**

| library | no. | | $X^4$ | $X^3$ | $X^2$ | $X^1$ |
|---|---|---|---|---|---|---|
| **AcL** | 1 | | His | Leu | Val | Lys |
| | 2 | **AcH1** | His | Phe | Arg | Lys |
| | 3 | | His | Gln | Ala | Lys |
| | 4 | **AcH2** | His | Gln | His | aPro |
| | 5 | | His | Val | Leu | Lys |
| | 6 | | His | Tyr | Arg | Lys |
| | 7 | | His | βAla | Ser | aPro |
| | 8 | **AcH3** | His | Leu | Val | Lys |
| | 9 | | His | Val | His | aPro |
| | 10 | | His | βAla | Thr | aPro |
| | 11 | **AcH4** | His | βAla | Arg | aPro |
| | 12 | | His | Gly | Val | aPro |
| | 13 | | Ser | Arg | His | aPro |
| | 14 | | His | Ala | Ala | aPro |
| | 15 | **AcH5** | Gly | His | His | aPro |
| | 16 | | His | | His | aPro |
| | 17 | | Arg | Leu | His | aPro |
| **L** | 18 | | His | Leu | Leu | Lys |
| | 19 | | Phe | His | Arg | aPro |
| | 20 | | Gln | Phe | His | aPro |
| | 21 | **H6** | His | Leu | Ala | Lys |
| | 22 | **H7** | His | Phe | Pro | aPro |
| | 23 | | Phe | His | Phe | aPro |
| | 24 | **H8** | His | bAla | Thr | aPro |
| | 25 | | Thr | His | His | aPro |
| | 26 | **H9** | His | | Gly | Lys |
| | 27 | | Pro | His | Arg | Lys |
| | 28 | **H10** | Phe | His | His | Lys |
| | 29 | | His | Phe | Val | Lys |
| | 30 | | Phe | His | Ala | aPro |
| | 31 | | His | Leu | Ser | aPro |
| | 32 | | Leu | His | His | aPro |
| | 33 | | His | | Ala | aPro |
| | 34 | | His | Leu | Pro | aPro |
| | 35 | | Phe | His | Ser | aPro |
| | 36 | | His | Arg | Arg | aPro |
| | 37 | | His | Phe | Ala | Lys |

Control and inactive beads showed approximately random amino acid composition. By contrast the sequences on active beads showed a strong consensus for histidine (Figure 3). All positive hits in libraries **AcL** and **L** had at least one histidine in the sequence, most frequently at the outer position $X^4$ (8 copies), such that the average amino acid composition comprised approximately 50% histidine. Histidine at position $X^4$ was generally combined with hydrophobic amino acids at the inner positions $X^3$ and $X^2$, in particular phenylalanine and leucine. The cationic arginine occurred in 9 of the 37 positive hits, but the anionic aspartate was totally absent from positive hits.

**Hit Resynthesis and Kinetic Studies.** Ten hits and four non-hits from screening with fluorecein diacetate **1** were selected equally from libraries **AcL** and **L** and resynthesized by SPPS (Table 3), using either Wang or Rink amide resin yielding a

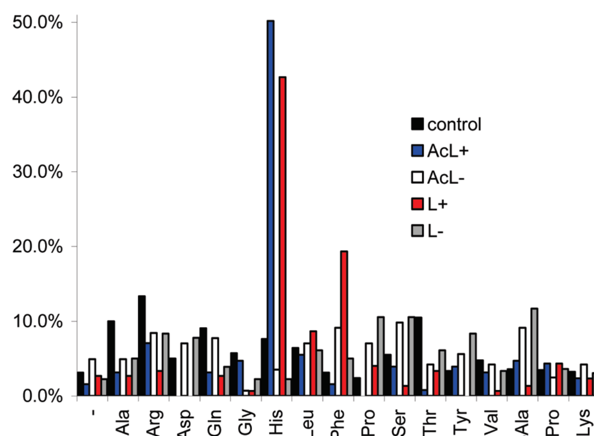



**Figure 3.** Composition of the dendrimer sequences. **Control**: beads picked randomly from library **L** (56 beads). **AcL+**: positive hits from **AcL** with fluorescein diacetate **1** (17 beads); **AcL-**: inactive beads from **AcL** with **1** (19 beads); **L+**: positive hits from **L** with **1** (20 beads); **L-**: inactive hits from **L** with **1** (24 beads). The total number of occurrences of each amino acid is calculated as $8m + 4n + 2p + q$ with $m$, $n$, $p$, and $q$ denoting the number of sequences with that amino acid at positions $X^4$, $X^3$, $X^2$, and $X^1$, respectively.

**Table 3. Synthesis of Hits and Non-Hit Peptide Dendrimers Identified by off-Bead Screening with Fluorescein Diacetate 1 with OBOC Libraries AcL and L**

| no. | sequence | yield, mg (%) | MS calc/ obs | MS obsd |
|---|---|---|---|---|
| **AcH1** | $(AcH)_8(KF)_4(KR)_2$ KKOH | 66.2 mg (18%) | 3376.8 | 3377 |
| **AcH2** | $(AcH)_8(KQ)_4$ $(KH)_2$KaPNH2 | 21.5 mg (8%) | 3245.7 | 3246 |
| **AcH3** | $(AcH)_8(KL)_4(KV)_2$KKOH | 50.3 mg (16%) | 3126.8 | 3127 |
| **AcH4** | $(AcH)_8$ $(K\beta A)_4(KR)_2$KaPNH2 | 2.4 mg (1%) | 3055.7 | 3056 |
| **AcH5** | $(AcG)_8(KH)_4(KH)_2$ KaPNH2 | 2.0 mg (1%) | 2641.4 | 2642 |
| **H6** | $(H)_8(KL)_4(KA)_2$KKOH | 62.0 mg (22%) | 2734.7 | 2735 |
| **H7** | $(H)_8(KF)_4(KP)_2KaPNH_2$ | 15.8 mg (7%) | 2905.6 | 2906 |
| **H8** | $(H)_8(K\beta A)_4(KT)_2KaPNH_2$ | 3.3 mg (2%) | 2609.5 | 2609 |
| **H9** | $(H)_8(K)_4(KG)_2$KKOH | 73.8 mg (29%) | 2254.3 | 2254 |
| **H10** | $(F)_8(KH)_4(KH)_2$KKOH | 64.0 mg (21%) | 3042.7 | 3043 |
| **AcN1** | $(AcS)_8(K)_4(KY)_2KaPNH_2$ | 7.9 mg (5%) | 2385.2 | 2385 |
| **AcN2** | $(AcF)_8(KR)_4(KH)_2$KKOH | 28.8 mg (9%) | 3454.9 | 3455 |
| **N3** | $(A)_8(KP)_4(KQ)_2KaPNH_2$ | 1.3 mg (1%) | 2239.4 | 2239 |
| **N4** | $(S)_8(KV)_4(KH)_2$KKOH | 46.5 mg (22%) | 2410.4 | 2410 |

C-terminal carboxyl or carboxamide (the nature of the C-terminus turned out to have negligible influence on catalysis). The dendrimers were obtained in good yields and purity after purification by RP-HPLC. The hit sequences carried 6 to 10 histidine residues. Two of the non-hits had no histidine residues and two had a pair of histidines at position $X^2$. A series of linear oligohistidine peptides from 1 to 15 residues (**His1-His15**) and the histidine-lysine oligomer **P25** ($AcHKHKHKHKHKHKHNH_2$), which were known to exhibit good esterolytic activity toward **4**,[15] were also included in the study. Kinetics were measured in 96-well microtiter plates following product formation by fluorescence in aqueous buffered solutions containing the peptide dendrimer at 5 $\mu M$ and the substrates **1**, **2** or **4**, including 17% acetonitrile cosolvent with **1** and 11% acetonitrile cosolvent with **2** (Table 4 and Supporting Information, Table S3, Figure 4).

Table 4. Kinetic Parameters for Dendrimer and Peptide Catalyzed Ester Hydrolysis

| no. | sequence | $N_{His}$ | $k_{cat}/K_M$ (1)[a] $M^{-1}$ $min^{-1}$ | $k_{cat}/K_M$ (2)[b] $M^{-1}$ $min^{-1}$ | $k_{cat}/K_M$ (4)[c] $M^{-1}$ $min^{-1}$ |
|---|---|---|---|---|---|
| **AcH1** | $(AcH)_8(KF)_4(KR)_2$ KKOH | 8 | 24 ± 2 | 112 ± 7 | 745 ± 30 |
| **AcH2** | $(AcH)_8(KQ)_4$ $(KH)_2$KaPNH$_2$ | 10 | 34 ± 8 | 160 ± 23 | 1100 ± 47 |
| **AcH3** | $(AcH)_8(KL)_4(KV)_2$KKOH | 8 | 25 ± 9 | 260 ± 70 | 910 ± 30 |
| **AcH4** | $(AcH)_8$ $(K\beta A)_4(KR)_2$KaPNH$_2$ | 8 | 19 ± 0.6 | 170 ± 20 | 1170 ± 230 |
| **AcH5** | $(AcG)_8(KH)_4(KH)_2$ KaPNH$_2$ | 6 | | 47 ± 5 | 1570 ± 110 |
| **H6** | $(H)_8(KL)_4(KA)_2$KKOH | 8 | 20 ± 5 | 33 ± 8 | 1770 ± 65 |
| **H7** | $(H)_8(KF)_4(KP)_2$KaPNH$_2$ | 8 | 47 ± 7 | 310 ± 70 | 3820 ± 260 |
| **H8** | $(H)_8(K\beta A)_4(KT)_2$KaPNH$_2$ | 8 | 35 ± 12 | 36 ± 8 | 3400 ± 80 |
| **H9** | $(H)_8(K)_4(KG)_2$KKOH | 8 | 21 ± 10 | 30 ± 3 | 2770 ± 26 |
| **H10** | $(F)_8(KH)_4(KH)_2$KKOH | 6 | 43 ± 19 | 130 ± 6 | 1690 ± 40 |
| **AcN1** | $(AcS)_8(K)_4(KY)_2$KaPNH$_2$ | | | | |
| **AcN2** | $(AcF)_8(KR)_4(KH)_2$KKOH | 2 | | 68 ± 9 | 670 ± 33 |
| **N3** | $(A)_8(KP)_4(KQ)_2$KaPNH$_2$ | | | | 137 ± 12 |
| **N4** | $(S)_8(KV)_4(KH)_2$KKOH | 2 | | 18 ± 5 | 660 ± 13 |
| **His1** | AcHNH$_2$ | 1 | nd | 5 ± 0.5 | 3 ± 0.2 |
| **His2** | AcHHNH$_2$ | 2 | nd | 10 ± 1 | 27 ± 0.4 |
| **His3** | AcHHHNH$_2$ | 3 | nd | 19 ± 1 | 132 ± 4 |
| **His4** | AcHHHHNH$_2$ | 4 | nd | 25 ± 2 | 220 ± 6 |
| **His5** | AcHHHHHNH$_2$ | 5 | 42 ± 14 | 36 ± 5 | 270 ± 7 |
| **His6** | AcHHHHHHNH$_2$ | 6 | 39 ± 12 | 35 ± 3 | 380 ± 2 |
| **His7** | AcHHHHHHHNH$_2$ | 7 | 36 ± 13 | 42 ± 3 | 796 ± 130 |
| **His8** | AcHHHHHHHHNH$_2$ | 8 | 43 ± 16 | 60 ± 7 | 960 ± 140 |
| **His9** | AcHHHHHHHHHNH$_2$ | 9 | 41 ± 20 | 65 ± 8 | 1010 ± 130 |
| **His10** | AcHHHHHHHHHHNH$_2$ | 10 | 56 ± 27 | 81 ± 5 | 1240 ± 110 |
| **His11** | AcHHHHHHHHHHHNH$_2$ | 11 | 49 ± 32 | 59 ± 2 | 1200 ± 130 |
| **His12** | AcHHHHHHHHHHHHNH$_2$ | 12 | nd | 70 ± 7 | 1540 ± 180 |
| **His13** | AcHHHHHHHHHHHHHNH$_2$ | 13 | 77 ± 46 | 75 ± 4 | 1570 ± 250 |
| **His14** | AcHHHHHHHHHHHHHHNH$_2$ | 14 | 83 ± 41 | 79 ± 3 | 1690 ± 220 |
| **His15** | AcHHHHHHHHHHHHHHHNH$_2$ | 15 | nd | 114 ± 13 | 2210 ± 190 |
| **P25** | AcHKHKHKHKHKHNH$_2$ | 6 | nd | 33 ± 3 | 2380 ± 200 |

[a] Conditions: 3.75−15 $\mu$M peptide or dendrimer, 3−100 $\mu$M **1**, 20 mM phosphate pH 7.6 with 17% acetonitrile, 34 °C. [b] Conditions: 3.75−20 $\mu$M peptide or dendrimer, 3−100 $\mu$M **2**, 5 mM citrate pH 5.5, with 11% acetonitrile, 34 °C. [c] Conditions: 3.75- 20 $\mu$M peptide or dendrimer, 58.5−1000 $\mu$M substrate **4**, 5 mM citrate pH 5.5, 34 °C. Spontaneous background reaction $k_{uncat}$ = 3.22 × $10^{-4}$ $min^{-1}$ for **1**, 3.37 × $10^{-5}$ $min^{-1}$ for **2**, 3.58 × $10^{-5}$ $min^{-1}$ for **4**. $k_2$ is the 2nd order rate constant with 4-methyl imidazole, $k_2$ =2.26 $M^{-1}$ $min^{-1}$ for **2**, $k_2$ = 1.0 $M^{-1}$ $min^{-1}$ for **4**. 120 $\mu$L assays in microtiterplate wells were followed by fluorescence ($\lambda_{exc}$ = 450 ± 25 nm, $\lambda_{em}$ = 530 ± 12 nm). All kinetics were run in triplicate. See Experimental Section and Supporting Information, Table S3 for complete data.

The hydrolysis of fluorescein diacetate **1** to fluorescein was investigated in phosphate buffer pH 7.6 in the presence of 17% acetonitrile cosolvent to ensure substrate solubility. Unfortunately experiments showed poor reproducibility and only provided an estimation of the apparent specificity constant $k_{cat}/K_M$. The data confirmed the screening results with all but one positive hit showing the expected activity and the four non-hit sequences showing no activity with this substrate. The apparent activity was comparable to that observed with linear oligohistidine peptides when counted per catalytic histidine residues.

To better characterize the unprecedented esterolytic activity of dendrimers with hydrophobic substrates, we prepared isobutyryl fluorescein **2** (Scheme 1). This substrate requires only a single ester bond cleavage to release the fluorescent product fluorescein, and was expected to show higher aqueous solubility because of the presence of a free carboxyl group. We also anticipated a better reproducibility of the kinetic data from our previous experiences with related straight-chain aliphatic fluorescein monoesters as lipase and esterase substrates.[16] Indeed substrate **2** gave better reproducible data than **1**, and showed activity both at pH 7.6 and at pH 5.5. The best kinetic data were collected at pH 5.5; however, the substrate concentration was held at 100 $\mu$M or below because of limited solubility, which precluded a determination of the Michaelis−Menten constant $K_M$. The data were therefore interpreted in terms of the specificity constant $k_{cat}/K_M$. The kinetic study was also carried out with the trianionic substrate **4** at the same pH because of its strong reactivity with histidine containing catalysts.

**Structure-Activity Relationships.** A comparative analysis of the catalytic proficiencies of the different peptide dendrimers with the hydrophobic ester substrate **2** and the polyanionic substrate **4** provided a useful insight into the structure-activity relationships. For each substrate the relative specificity constants compared to the reference small molecule catalyst 4-methylimidazole were computed as $(k_{cat}/K_M)/k_2(\mathbf{2})$ and $(k_{cat}/K_M)/k_2(\mathbf{4})$, respectively $((k_{cat}/K_M)/k_2)/N_{His}(\mathbf{2})$ and $((k_{cat}/K_M)/k_2)/N_{His}(\mathbf{4})$ for the effect counted per histidine residue (Figure 5A and 5B).

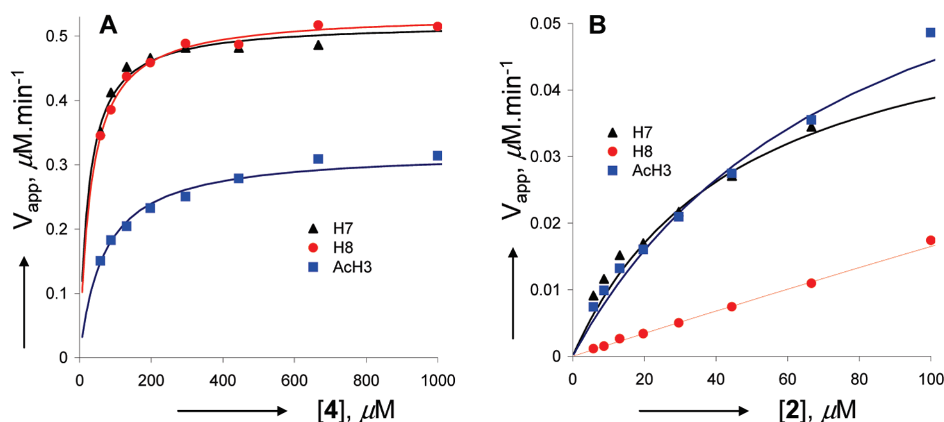



**Figure 4.** Catalytic reaction rates with peptide dendrimers **H7**, **H8**, and **AcH3**. Conditions: aq. 5 mM citrate pH 5.5 with 5 $\mu$M dendrimer.

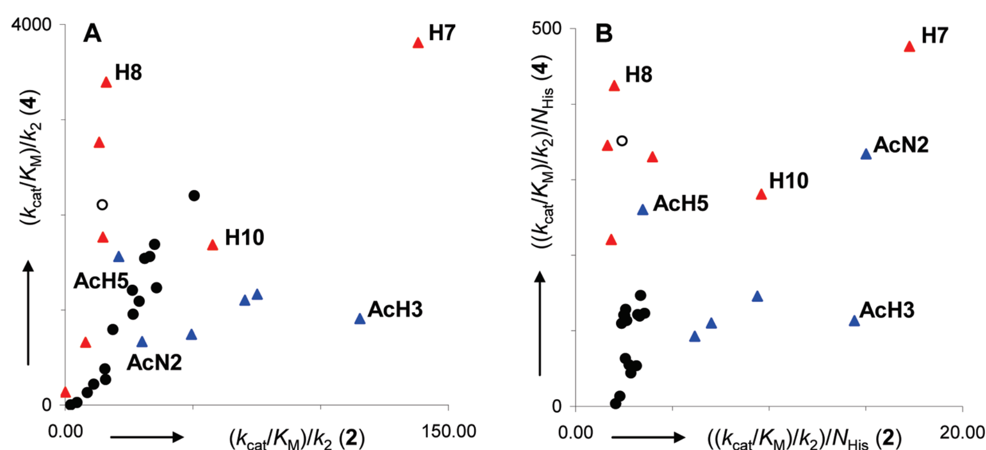



**Figure 5.** Relative catalytic proficiencies of histidine containing peptides and peptide dendrimers with substrates **2** and **4** in aqueous 5 mM citrate buffer pH 5.5. (**A**) Relative catalytic proficiencies $(k_{cat}/K_M)/k_2$. $k_2$ is the 2nd order rate constant with 4-methyl-imidazole as reference catalyst. (**B**) Relative catalytic proficiencies per histidine residue $((k_{cat}/K_M)/k_2)/N_{His}$. $N_{His}$ is the number of histidine residue in the peptide or peptide dendrimer catalyst. (red solid triangles): peptide dendrimers from library **L**; (blue solid triangles): peptide dendrimers from library **AcL**; (black solid circles): oligohistidine peptide from 1 to 15 amino acid residues, with N-terminal acetylation. (black open circles): octapeptide AcHKHKHKHKNH$_2$. See Table 4 for detailed kinetic data and conditions.

The reference oligohistidine peptides **His1-His15** showed increasing relative catalytic proficiencies $(k_{cat}/K_M)/k_2$ as a function of length for both substrates. The relative catalytic proficiencies for substrate **4** were on average 40-fold larger than for substrate **2**. The catalytic effect per histidine residue for the trianionic substrate **4** $((k_{cat}/K_M)/k_2)/N_{His}(4)$ strongly increased up to seven histidine residues before leveling off. By contrast, the catalytic proficiency per histidine residue for the isobutyryl fluorescein substrate **2** $((k_{cat}/K_M)/k_2)/N_{His}(2)$ was essentially independent of peptide length. Both the higher catalytic activity with **4** compared to **2** and the length dependence of $((k_{cat}/K_M)/k_2)/N_{His}(4)$ may be explained by significant electrostatic binding with substrate **4** requiring cooperative action of several histidine residues, while the more hydrophobic substrate **2** does not bind and is therefore insensitive to peptide length.

The peptide dendrimers showed a differentiated catalytic behavior toward **2** and **4**, with several dendrimers standing out by their reactivities, in particular relative to the linear peptides. Dendrimers with acetylated N-termini from library **AcL** were generally more active toward the hydrophobic fluorescein substrate **2**. The most selective catalyst for substrate **2** was peptide dendrimer **AcH3** (AcHis)$_8$(*Lys*Leu)$_4$(*Lys*Val)$_2$*Lys*LysOH, which combined eight histidine residues in the third generation branches with hydrophobic residues in the first and second generation branches. On the other hand peptide dendrimer **AcH5** (AcGly)$_8$(*Lys*His)$_4$(*Lys*His)$_2$ *Lys*aProNH$_2$ with six histidine residues in the inner first and second generation branches and only glycine residue in the third generation branch was more selective toward the trianionic substrate **4**. Interestingly the negative control dendrimer **AcN2** (AcPhe)$_8$(*Lys*Arg)$_4$-(*Lys*His)$_2$*Lys*LysOH, which was not active with fluorescein diacetate **1**, showed significant activity with the isobutyrate **2** as well as with the trisulfonate **4**. This reactivity stood out in particular when considering the specific catalytic effect per histidine residue, and probably reflects substrate binding interactions with the arginine side chains.

The peptide dendrimers from library **L** with free N-termini were more reactive toward the trianionic substrate **4**, in particular for dendrimer **H8** (His)$_8$(*Lys*$\beta$Ala)$_4$(*Lys*Thr)$_2$*Lys*aProNH$_2$, reflecting electrostatic substrate binding. This dendrimer showed only poor reactivity with **2** attributable to the absence of

hydrophobic residues. Peptide dendrimer **H7** $(His)_8(LysPhe)_4(LysPro)_2Lys$aProNH$_2$ on the other hand had the strongest reactivity of all catalysts toward both substrates. A particularly favorable combination of eight catalytic histidine residues at the N-terminus with multiple positive charges from the free N-termini favored activity with **4.** In addition the hydrophobic interior of **H7** constituted of four phenylalanines in the second generation branch and two proline residues in the first generation branch favored activity with **2**. Peptide dendrimer **H10** $(Phe)_8(LysHis)_4(LysHis)_2Lys$LysOH featuring an inverted combination with eight hydrophobic phenylalanines in the third generation branches and six catalytic histidines in the inner branches showed a weaker but similarly balanced reactivity between **2** and **4**. By comparison the linear peptide **P25** AcHKHKHKHKHKHKHNH$_2$ which combines six histidine residues with five ammonium group from lysine side chains showed only enhanced catalysis with the trisulfonate **4** but not with isobutyryl fluorescein **2**, because of the absence of hydrophobic residues.

**Mass Spectrometric Analysis.** As previously observed with **P25**, the peptide dendrimers from library **L** underwent significant acylation of the N-termini in contact with substrate **4**, as detected by MS for the reaction of **H9** and **H10** under high substrate and dendrimer concentration (see Supporting Information). In the case of dendrimer **H9** MS analysis also showed the formation of substrate-dendrimer complexes with one, two, and three substrates per dendrimer, in agreement with the multivalent nature of these catalysts. On the other hand acylation with isobutyrate **2** occurred only to a very low level, probably because of the slower reaction rates and the bulkier acyl group in that substrate.

## CONCLUSION

The experiments above showed the first example of a combinatorial peptide dendrimer library extensively covering its sequence space by using 14 amino acids and a deletion at each variable position, exploiting the fact that such exhaustive sampling is compatible with AAA for bead decoding. Screening of catalysis with fluorogenic substrates in the off-bead *in silica* assay uncovered specific reactivities with fluorescein diacetate **1**. While this substrate proved unsuitable for kinetic studies, the related isobutyryl fluorescein **2**, which requires only a single ester bond cleavage to release its fluorescent product, allowed the characterization of this reactivity. Structure-activity relationship studies comparing the catalyzed ester hydrolysis reaction rates of **2** with those of the trianionic substrate **4** showed that efficient hydrolysis of the hydrophobic substrate **2** requires a combination of multiple catalytic histidine residues with hydrophobic amino acids such as phenylalanine and leucine. These hydrophobic residues presumably enable substrate binding, although pre-equilibrium binding was not visible in the accessible concentration range. By contrast peptide dendrimers with mostly cationic residues and no hydrophobic groups showed stronger activity with the trisulfonate **4**. These experiments highlight the power of combinatorial approaches to identify peptidic enzyme models and the role of noncatalytic amino acids to determine substrate selectivity in these systems.

## EXPERIMENTAL SECTION

**General Procedures.** All reagents were either purchased from Aldrich, Fluka, or Acors Organics. PyBOP, amino acids, and their derivatives were purchased from Advanced ChemTech (U.S.A.) or Novabiochem (Switzerland). Amino acids were used as the following derivatives: Fmoc-Ala-OH, Fmoc-$\beta$Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-His(Boc)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(Fmoc)-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Val-OH. Fmoc-$^{a}$Pro(Boc)-OH was purchased from Aldrich. TG S RAM (loading: 0.23 or 0.24 mmol·g$^{-1}$) resin was purchased from Rapp Polymere (Germany) while hydroxymethyl-photolinker NovaSyn TG (loading: 0.24 mmol·g$^{-1}$) and TGA (loading: 0.26 mmol·g$^{-1}$) were purchased from Novabiochem. Peptide dendrimer syntheses were performed manually in polypropylene syringes fitted with a polyethylene frit, a Teflon stopcock and stopper. Analytical RP-HPLC was performed in Waters (996 Photo diode array detector) chromatography system using Atlantis column (dC18, 5 $\mu$m, 4.6 × 100 mm, flow rate 1.4 mL·min$^{-1}$). Analytical RP-UPLC was performed by Ultimate 3000 Rapid Separation LC System using diode array detector (DAD-3000RS) and Acclaim RSLC 120 C18 column (2.2 $\mu$m, 120 Å, 3.0 × 50 mm, flow 1.2 mL min$^{-1}$) from Dionex. Preparative RP-HPLC was performed in Waters Prep LC4000 chromatography system using a Delta-Pak column (C18, 15 $\mu$m, pore size 300 Å, flow rate 80 mL·min$^{-1}$) or an Atlantis OBD column (dC18, 5 $\mu$m, 19 × 100 mm, flow rate 20 mL·min$^{-1}$). Compounds were detected by UV absorption at 214 nm. All RP-HPLC were using HPLC-grade acetonitrile and mQ-deionized water. The elution solutions were **A** $H_2O$ with 0.1% TFA; **B** $H_2O$/MeCN (50:50); **C** $H_2O$/MeCN (10:90) with 0.1% TFA; **D** $H_2O$/MeCN (40:60) with 0.1% TFA. MS spectra and amino acid analyses measurements were provided by Mass Spectrometry, and Protein Analysis services respectively of the Department of Chemistry and Biochemistry at the University of Bern. Kinetic measurements were carried out using a CytoFluor Series 4000 multiwell plate reader from PerSeptive Biosystems.

**Synthesis of Split-and-Mix Library.** The peptide dendrimer library was prepared from 1.5 g−100 mg per split-resin batch of hydroxymethyl-photolinker NovaSyn TG resin (0.24 mmol·g$^{-1}$) divided equally in 15 reactors. The attachment of the first amino acid to hydroxymethyl resin was done by using MSNT with 1-MeIm.[17] The resin was placed in a syringe and swelled with dry $CH_2Cl_2$ under nitrogen. In a round-bottom flask, the appropriate amino acid (5 equiv) was dissolved in dry $CH_2Cl_2$ (3 mL per mmol) and a few drops of tetrahydrofuran (THF). MSNT (5 equiv) and 1-methylimidazole (3.75 equiv) were added, dissolved, and the mixture was added to the syringe. Then the syringe was agitated at RT for 1 h. The resin was washed with $CH_2Cl_2$ (5 × 5 mL). The coupling was repeated two times. A sensitive color test (alizarin-cyanuric chloride test)[18] was used to detect the presence of hydroxyl groups on solid support; the presence of unreacted hydroxyl groups was indicated by red-colored beads. The Fmoc-protecting groups of the resin were removed with a solution of 20% piperidine in dimethylformamide (DMF, 2 × 10 min). For further couplings, the resin was acylated with one of the amino acid (3 eq/G, G = generation) in the presence of PyBOP (3 eq/G) and DIEA (5 eq/G) in NMP.[3c] Amino acids, derivatives or diamino acids were coupled for 30 min (G0), 1 h (G1), 2 h (G2), 4 h (G3). The completion of the reaction was checked using 2,4,6-trinitrobenzenesulfonic acid solution (TNBS) or chloranil test.[19] If the beads were red (brown for proline), there were some free amino

groups and the resin test was positive. If they were colorless, there were no more free amino groups, and the resin test was negative. The coupling was repeated after a positive test. After each coupling, the resin in each syringe was deprotected (20% piperidine in DMF, 2 × 10 min) followed by TNBS or chloranil test (test must be positive). Then the resin batches were mixed together, vortexed during 1 min, and split equally. These split-and-mix steps were repeated after each amino acid coupling. After each coupling or deprotection, the resin was successively washed with NMP, MeOH, and $CH_2Cl_2$ (3 × 5 mL, with each solvent). At the end of the synthesis, the Fmoc protected library was dried and stored at −18 °C. For all the steps, the syringes were covered with aluminum to prevent exposure to light.

**Off-Bead Screening.** Before the screening of the library, the Fmoc protecting groups were removed and the last amino acid was acetylated with $Ac_2O/CH_2Cl_2$ (1:1) for 1 h (library **AcL**) or not acetylated (library **L**). The side-chain protecting groups were removed with TFA/TIS (triisopropylsilane)/$H_2O$ (94:5:1) during 4 h, resulting in a functional dendrimer library on-beads. Screening was performed by washing the beads (5 × 3 mL) with the buffer and swelling those (10 mg, 2 × 1 h) in the same aqueous buffered solution. After solution filtration and drying under vacuum, the resin was poured on a Petri dish (diameter: 3 cm) and was partially irradiated under an Hg lamp (100 W) with a 366 nm filter for 30 min. A glass TLC plate (10 × 10 cm, SIL G25 without fluorescent indicator) was impregnated with 100 μM fluorescein diacetate **1** dissolved in $CH_2Cl_2$, and dried. The TLC was sprayed with PB buffer (60 mM, pH 7.6) and the photolyzed beads were poured on the slightly wet TLC and incubated in a close Petri dish for 12 h. Under UV irradiation (366 nm) a green fluorescent halo appeared on the TLC around some of the beads. These active beads were picked and subjected to AAA.

**Sequence Determination.** Single dendrimer-containing resin beads were hydrolyzed with 6 M aqueous HCl at 110 °C for 22 h. The amino acids were derivatized with phenylisothiocyanate (PITC) and the phenylthiocarbamyl (PTC) derivatives analyzed on a RP-C18 Novapack column.

**Dendrimer Synthesis.** The resin (TG S RAM, or TGA) was swelled in $CH_2Cl_2$, and the Fmoc-protecting groups of the resin were removed with a solution of 20% piperidine in DMF (2 × 10 min). Then the resin was acylated with each amino acid, derivative, or diamino acid (3 eq/G) using PyBOP (3 eq/G) and DIEA (5 eq/G) in NMP. The first coupling on the TGA resin used the described protocol (MSNT, 1-MeIm). The completion of the reaction was checked using the TNBS or chloranil test (alizarin-cyanuric chloride test for the first coupling on TGA). At the end of the synthesis, the resin was acetylated with $Ac_2O/CH_2Cl_2$ (1:1) for 1 h for library **AcL**. The cleavage was carried out with TFA/TIS/$H_2O$ (94:5:1) during 4 h. The peptide was precipitated with *tert*-butylmethylether (MTBE) then dissolved in a $H_2O$/MeCN mixture. Peptides were purified by preparative RP-HPLC and obtained as TFA salts after lyophilization. Unless otherwise mentioned, the gradient used for analytical HPLC is $A/D$ = 100/0 to 0/100 in 10 min, 100 D in 5 min, 1.4 mL·min$^{-1}$.

*(AcHis)$_8$(LysPhe)$_4$(LysArg)$_2$LysLysOH* ***(AcH1)***. Starting with 300 mg of TGA (0.26 mmol·g$^{-1}$), dendrimer **AcH1** was obtained as a white foamy solid after cleavage from the resin and preparative RP-HPLC purification (66.2 mg, 18%). Anal. RP-HPLC: $t_R$ = 8.1 min. MS (ES+) calcd for $C_{160}H_{231}N_{52}O_{31}$ $[M+H]^+$: 3376.8, found: 3377.0; $[M+K]^+$: 3414.8, found: 3416.0; $[M+TFA]^+$: 3488.8, found: 3491.0.

*(AcHis)$_8$(LysGln)$_4$(LysHis)$_2$Lys$^a$ProNH$_2$* ***(AcH2)***. Starting with 250 mg of TG S RAM (0.24 mmol·g$^{-1}$), dendrimer **AcH2** was obtained as a white foamy solid after cleavage from the resin and preparative RP-HPLC purification (21.5 mg, 8%). Anal. RP-HPLC: $t_R$ = 7.0 min. MS (ES+) calcd for $C_{143}H_{214}N_{55}O_{34}$ $[M+H]^+$: 3245.7, found: 3246.0; $[M+K]^+$: 3287.7, found: 3285.0.

*(AcHis)$_8$(LysLeu)$_4$(LysVal)$_2$LysLysOH* ***(AcH3)***. Starting with 300 mg of TGA (0.26 mmol·g$^{-1}$), dendrimer **AcH3** was obtained as a white foamy solid after cleavage from the resin and preparative RP-HPLC purification (50.3 mg, 16%). Anal. RP-HPLC: $t_R$ = 8.3 min. MS (ES+) calcd for $C_{146}H_{233}N_{46}O_{31}$ $[M+H]^+$: 3126.8, found: 3127.0; $[M+Na]^+$: 3149.8, found: 3149.0; $[M+K]^+$: 3165.8, found: 3167.0.

*(AcHis)$_8$(LysβAla)$_4$(LysArg)$_2$Lys$^a$ProNH$_2$* ***(AcH4)***. Starting with 250 mg of TG S RAM (0.24 mmol·g$^{-1}$), dendrimer **AcH4** was obtained as a white foamy solid after cleavage from the resin and preparative RP-HPLC purification (2.4 mg, 1%). Anal. RP-HPLC: $t_R$ = 6.8 min. MS (ES+) calcd for $C_{135}H_{212}N_{53}O_{30}$ $[M+H]^+$: 3055.7, found: 3056.0; $[M+K]^+$: 3093.7, found: 3097.0.

*(AcGly)$_8$(LysHis)$_4$(LysHis)$_2$Lys$^a$ProNH$_2$* ***(AcH5)***. Starting with 250 mg of TG S RAM (0.24 mmol·g$^{-1}$), dendrimer **AcH5** was obtained as a white foamy solid after cleavage from the resin and preparative RP-HPLC purification (2.0 mg, 1%). Anal. RP-HPLC: $t_R$ = 7.0 min. MS (ES+) calcd for $C_{115}H_{178}N_{43}O_{30}$ $[M+H]^+$: 2641.4, found: 2642.0; $[M+K]^+$: 2679.4, found: 2682.0; $[M+TFA]^+$: 2753.4, found: 2756.0.

*(His)$_8$(LysLeu)$_4$ (LysAla)$_2$LysLysOH* ***(H6)***. Starting with 300 mg of TGA (0.26 mmol·g$^{-1}$), dendrimer **H6** was obtained as a white foamy solid after cleavage from the resin and preparative RP-HPLC purification (62.0 mg, 22%). Anal. RP-UPLC: $t_R$ = 1.312 min ($A/D$ = 100/0 to 0/100 in 2.2 min, 100 D in 1.3 min, $A/D$ = 0/100 to 100/0 in 0.5 min, 100 A in 1 min 1.2 mL·min$^{-1}$). MS (ES+) calcd for $C_{126}H_{209}N_{46}O_{23}$ $[M+H]^+$: 2734.7, found: 2735.0; $[M+K]^+$: 2773.7, found: 2775.0.

*(His)$_8$(LysPhe)$_4$(LysPro)$_2$Lys$^a$ProNH$_2$* ***(H7)***. Starting with 250 mg of TG S RAM (0.24 mmol·g$^{-1}$), dendrimer **H7** was obtained as a white foamy solid after cleavage from the resin and preparative RP-HPLC purification (15.8 mg, 7%). Anal. RP-HPLC: $t_R$ = 7.7 min. MS (ES+) calcd for $C_{141}H_{202}N_{47}O_{22}$ $[M+H]^+$: 2905.6, found: 2906.0; $[M+K]^+$: 2943.6, found: 2946.0.

*(His)$_8$(LysβAla)$_4$(LysThr)$_2$Lys$^a$ProNH$_2$* ***(H8)***. Starting with 250 mg of TG S RAM (0.24 mmol·g$^{-1}$), dendrimer **H8** was obtained as a white foamy solid after cleavage from the resin and preparative RP-HPLC purification (3.3 mg, 2%). Anal. RP-HPLC: $t_R$ = 7.0 min. MS (ES+) calcd for $C_{115}H_{186}N_{47}O_{24}$ $[M+H]^+$: 2609.5, found: 2609.0.

*(His)$_8$(Lys)$_4$(LysGly)$_2$LysLysOH* ***(H9)***. Starting with 300 mg of TGA (0.26 mmol·g$^{-1}$), dendrimer **H9** was obtained as a white foamy solid after cleavage from the resin and preparative RP-HPLC purification (73.8 mg, 29%). Anal. RP-UPLC: $t_R$ = 1.212 min ($A/D$ = 100/0 to 0/100 in 2.2 min, 100 D in 1.3 min, $A/D$ = 0/100 to 100/0 in 0.5 min, 100 A in 1 min 1.2 mL·min$^{-1}$). MS (ES+) calcd for $C_{100}H_{161}N_{42}O_{19}$ $[M+H]^+$: 2254.3, found: 2254.0; $[M+Na]^+$: 2276.3, found: 2277.0; $[M+K]^+$: 2293.3, found: 2295.0.

*(Phe)$_8$(LysHis)$_4$(LysHis)$_2$LysLysOH* ***(H10)***. Starting with 300 mg of TGA (0.26 mmol·g$^{-1}$), dendrimer **H10** was obtained as a white foamy solid after cleavage from the resin and preparative RP-HPLC purification (64.0 mg, 21%). Anal. RP-HPLC:

$t_R$ = 7.3 min ($A/D$ = 100/0 to 0/100 in 7 min, 100 D in 3 min, 1.4 mL·min$^{-1}$). MS (ES+) calcd for $C_{156}H_{213}N_{42}O_{23}$ $[M+H]^+$: 3042.7, found: 3043.0; $[M+K]^+$: 3082.7, found: 3083.0.

*(AcSer)$_8$(Lys)$_4$(LysTyr)$_2$Lys$^a$ProNH$_2$* ***(AcN1)***. Starting with 250 mg of TG S RAM (0.24 mmol·g$^{-1}$), dendrimer **AcN1** was obtained as a white foamy solid after cleavage from the resin and preparative RP-HPLC purification (7.9 mg, 5%). Anal. RP-HPLC: $t_R$ = 7.7 min. MS (ES+) calcd for $C_{105}H_{170}N_{27}O_{36}$ $[M+H]^+$: 2385.2, found: 2385.0; $[M+Na]^+$: 2407.2, found: 2407.0; $[M+K]^+$: 2423.2, found: 2423.0.

*(AcPhe)$_8$(LysArg)$_4$(LysHis)$_2$LysLysOH* ***(AcN2)***. Starting with 300 mg of TGA (0.26 mmol·g$^{-1}$), dendrimer **AcN2** was obtained as a white foamy solid after cleavage from the resin and preparative RP-HPLC purification (28.8 mg, 9%). Anal. RP-UPLC: $t_R$ = 1.777 min ($A/D$ = 100/0 to 0/100 in 2.2 min, 100 D in 1.3 min, $A/D$ = 0/100 to 100/0 in 0.5 min, 100 A in 1 min, 1.2 mL·min$^{-1}$). MS (ES+) calcd for $C_{172}H_{249}N_{46}O_{31}$ $[M+H]^+$: 3454.9, found: 3455.0; $[M+TFA]^+$: 3567.9, found: 3570.0.

*(Ala)$_8$(LysPro)$_4$(LysGln)$_2$Lys$^a$ProNH$_2$* ***(N3)***. Starting with 250 mg of TG S RAM (0.24 mmol·g$^{-1}$), dendrimer **N3** was obtained as a white foamy solid after cleavage from the resin and preparative RP-HPLC purification (1.3 mg, 1%). Anal. RP-HPLC: $t_R$ = 6.6 min. MS (ES+) calcd for $C_{101}H_{180}N_{33}O_{24}$ $[M+H]^+$: 2239.4, found: 2239.0; $[M+K]^+$: 2277.4, found: 2278.0.

*(Ser)$_8$(LysVal)$_4$(LysHis)$_2$LysLysOH* ***(N4)***. Starting with 300 mg of TGA (0.26 mmol·g$^{-1}$), dendrimer **N4** was obtained as a white foamy solid after cleavage from the resin and preparative RP-HPLC purification (46.5 mg, 22%). Anal. RP-HPLC: $t_R$ = 6.9 min. MS (ES+) calcd for $C_{104}H_{189}N_{34}O_{31}$ $[M+H]^+$: 2410.4, found: 2410.0; $[M+Na]^+$: 2432.4, found: 2432.0; $[M+K]^+$: 2449.4, found: 2450.0.

*Isobutyryl Fluorescein (**2**)*. Fluorescein (500 mg. 1.5 mmol) was added to a suspension of 43 mg NaH (1.2 equiv.) and 50 mg $ZnCl_2$ (0.25 equiv.) in 10 mL of anhydrous DMF in the presence of molecular sieves. Isobutyryl chloride (160 $\mu$L, 1 equiv.) in 0.25 mL of dry DMF was added dropwise to the first solution for 5 min. After 18 h the reaction mixture was poured into 100 mL of ethyl acetate, washed with brine (3 × 100 mL), and dried over $Na_2SO_4$. The solvents were removed, and the crude was purified with flash chromatography (hexane-ethyl acetate 6:4)) to yield 233 mg of pure **2.** Yield 38%.$^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ = 8.04−7.99 (m, 1H), 7.69−7.56 (m, 2H), 7.11−7.04 (m, 2H), 6.83−6.72 (m, 2H), 6.69 (d, $J$ = 2.3 Hz, 1H), 6.55 (dt, $J$ = 8.7, 5.5 Hz, 2H), 2.83 (dt, $J$ = 17.7, 7.0 Hz, 1H), 1.33 (d, $J$ = 1.2 Hz, 3H), 1.31 (d, $J$ = 1.2 Hz, 3H). $^{13}$C NMR ($CDCl_3$, 400 MHz) $\delta$ = 175.8, 170.1, 158.7, 152.9, 152.6, 152.5, 152.2, 135.4, 130.1, 129.5, 126.7, 125.5, 124.4, 117.6, 116.8, 113.1, 110.9, 110.6, 103.4, 34.5, 19.1. Hrms (ESI+) Calculated for $C_{24}H_{19}O_6$ $[M+H]^+$, 403.1181, observed 403.1176, $[M+Na]^+$ 426.39, observed 425.10.

**Kinetic Measurements.** Peptides solutions were used as 11.5 $\mu$M (His10-His14), 15 $\mu$M (all dendrimers), 22.5 $\mu$M (His8-His9), 45 $\mu$M (His5-His7) for substrate **1**, 11.5 $\mu$M (His11-His15, P25), 15 $\mu$M (all dendrimers), 22.5 $\mu$M (His8-His10), 45 $\mu$M (His5-His7), 60 $\mu$M (His1-His4) for substrate **2**, 11.5 $\mu$M (His15, P25), 15 $\mu$M (all dendrimers, His7-His14), 22.5 $\mu$M (His8-His10), 45 $\mu$M (His5-His6), 60 $\mu$M (His1-His4) for substrate **4**, as freshly prepared solutions in mQ-$H_2O$. Phosphate buffer 60 mM pH 7.6, citric acid-sodium citrate buffer 15 mM at pH 5.5 were used as buffer, and the pH was adjusted to the desired value with HCl 1.0 M or NaOH 1.0 M using a Metrohm 692 pH/ion meter. Eight final substrate concentrations ranging from 5.8 to 100 $\mu$M in MeCN/buffer (17:83) for substrate **1**, 5.8 to 100 $\mu$M in MeCN/buffer (11:89) for substrate **2**, 58.5 to 1000 $\mu$M in buffer for substrate **4** (dilutions by 2/3) were measured for each Michaelis−Menten plot. Eight solutions of fluorescein sodium salt (FNa) ranging from 0 to 10 $\mu$M in in MeCN/buffer (17:83) for substrate **1**, in MeCN/buffer (11:89) for substrate **2**, 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt (HPTS) from 0 to 100 $\mu$M in buffer for substrate **4** were used for the calibration curve. A 40 $\mu$L portion of dendrimer was mixed with 40 $\mu$L of buffer and 40 $\mu$L of substrate in a 96-well half area flat bottom plate (190 $\mu$L). The formation of FNa or HPTS was followed by fluorescence emission using absorbance filter 450/50 and emission filter 530/25. The calibration curve and the blank (40 $\mu$L substrate, 40 $\mu$L buffer and 40 $\mu$L mQ-$H_2O$) were recorded for every experiment in parallel. Prior to every experiment the gain was adjusted to a signal of ∼50000 for the maximum concentration of FNa (for **1** nd **2**) or HPTS (for **4**) well. The temperature inside the instrument was set to 34 °C. Kinetic experiments were followed for typically 1−3 h, and the data points were measured every 90 s. Fluorescence data were converted into product concentration by means of the calibration curve. Initial reaction rates were calculated from the steepest linear part observed in the curve that gives fluorescence versus time.

**Kinetic Parameters $k_{cat}$ and $K_M$.** $V_{cat}$ is the apparent rate in the presence of dendrimer catalyst, and $V_{uncat}$ is the rate in buffer alone. The observed rate enhancement is defined as $V_{net}/V_{uncat}$ with $V_{net} = V_{cat} - V_{uncat}$. Michaelis−Menten parameters $k_{cat}$ (rate constant) and $K_M$ (Michaelis constant) were obtained by fitting the data to the Michaelis−Menten model. With substrate **2** where saturation was not reached no determination of $k_{cat}$ and $K_M$ was possible and only the specificity constant $k_{cat}/K_M$ is given. The rate constant $k_{uncat}$ without catalyst was calculated from the slope of the linear curve that gives $V_{uncat}$ (as product concentration per time) versus substrate concentration [S].

***$k_2$*** *(4-Methylimidazole).* The solutions of 4-methylimidazole were prepared by serial dilution from a stock solution (3 mM) adjusted to the desired pH value using HCl 1 M. The reaction rate with 4-methylimidazole was obtained under the same conditions as described above. The final concentrations in the plate were 0, 88, 132, 198, 296, 444, 667 and 1000 $\mu$M of 4-MeIm, 200 $\mu$M of substrate, and 60 mM of buffer. The second order rate constants $k_2$ were calculated from linear regression of the experimentally measured pseudo first order rate constants $k_2'$ as a function of 4-methylimidazole concentrations. The second order rate constants $k_2$ is given by $k_2 = k_2'/[S]$.

## ■ ASSOCIATED CONTENT

**Supporting Information.** Tables S1−S3, catalytic dendrimers (HPLC traces, MS and kinetics spectra). This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

### Corresponding Author

*Fax: + 41 31 631 80 57. E-mail: jean-louis.reymond@ioc.unibe.ch.

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