Anthony Clouet
Tamis Darbre
Jean-Louis Reymond
Department of Chemistry and Biochemistry, University of Berne, Freiestrasse 3, CH-3012 Berne, Switzerland

Combinatorial Synthesis, Selection, and Properties of Esterase Peptide Dendrimers

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Abstract: *A 65,536-member combinatorial library of peptide dendrimers was prepared by split-and-mix synthesis and screened on solid support for esterolytic activity in aqueous buffer using 8-butyryloxypyrene-1,3,6-trisulfonate (**2**) as a fluorogenic substrate. Active sequences were identified by analysis of fluorescent beads. The corresponding dendrimers were resynthesized by solid-phase synthesis, cleaved from the resin, and purified by preparative reverse-phase HPLC. The dendrimers showed the expected catalytic activity in aqueous buffer. Catalysis was studied against a pannel of fluorogenic 8-acyloxypyrene-1,3,6-trisulfonate substrates. The catalytic peptide dendrimers display enzyme-like kinetics in aqueous buffer with substrate binding in the range* $K_M \sim 0.1$ *m*M, *catalytic rate constants* $k_{cat} \sim 0.1\ min^{-1}$, *and specific rate accelerations over background up to* $k_{cat}/k_{uncat} = 10{,}000$. © 2005 Wiley Periodicals, Inc. Biopolymers 84: 114–123, 2006

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INTRODUCTION

Dendrimers are ramified tree-like molecules currently under study in several areas of chemistry that display a range of special properties and functions.[1] We are investigating peptide dendrimers constituted of alternating sequences of proteinogenic α-amino acid and diamino acid building blocks as artificial protein models for catalysis[2] and drug delivery.[3] Natural proteins are the products of evolution, which selects functional sequences from a large genetically encoded repertoire of linear amino acid combinations by mutation and selection steps. Due to the linear nature of polypeptides, natural evolution must select amino acid sequences for both folding and function. In our approach with dendrimers, the branched topology circumvents the protein folding problem since any dendritic sequence must adopt a globular or disk-shaped structure. Obtaining productive interactions between amino acids within the dendritic topology however remains a complex problem not very different from the situation within a folded protein, where small amino acid modifications often strongly affect function without significantly disturbing structure. Our peptide dendrimer approach to artificial proteins incorporates a combinatorial component to systematically search active sequences in combinations preselected by the choice and placement of amino acids

Correspondence to: J.-L. Reymond; e-mail; jean-louis.reymond@ioc.unibe.ch

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within the dendrimer. This combinatorial strategy should mimic natural evolution and facilitate the discovery of functional peptide dendrimers.

In initial studies we identified esterase-like peptide dendrimers in small libraries of peptide dendrimers composed of the catalytic triad amino acids aspartate, histidine, and serine in alternance with a branching diamino acid.[4] This combinatorial strategy was however limited because the peptide dendrimers were obtained by disulfide-bond dimerization in solution, which required an individual reaction and purification step of each dendrimer.

Herein we report a combinatorial approach to peptide dendrimers based on the split-and-mix strategy, and the selection of active esterase dendrimers from a 65,536-member library by direct fluorescence screening for activity on solid support.[5] Split-and-mix synthesis makes possible the generation of a large number of compounds with only a few synthetic steps.[6] The method generates single beads displaying only one type of compound and can be applied for the screening of millions of different compounds while still attached to the beads. The method has been used to identify peptides that interact specifically with antibodies, enzymes, artificial receptors, organic dyes, and other molecules as well as in the synthesis of small molecule catalysts.[7] The present study shows that split-and-mix synthesis allows the discovery of catalytically active sequences in peptide dendrimers. Direct screening of the solid-supported library is demonstrated with soluble substrates. The dendrimer combinatorial library is self-encoded and can be analyzed by amino acid composition analysis, which obviates the need for encoding. This favorable experimental setting combined with the use of commercially available amino acid building blocks makes peptide dendrimers libraries particularly attractive for combinatorial supramolecular chemistry.

RESULTS AND DISCUSSION

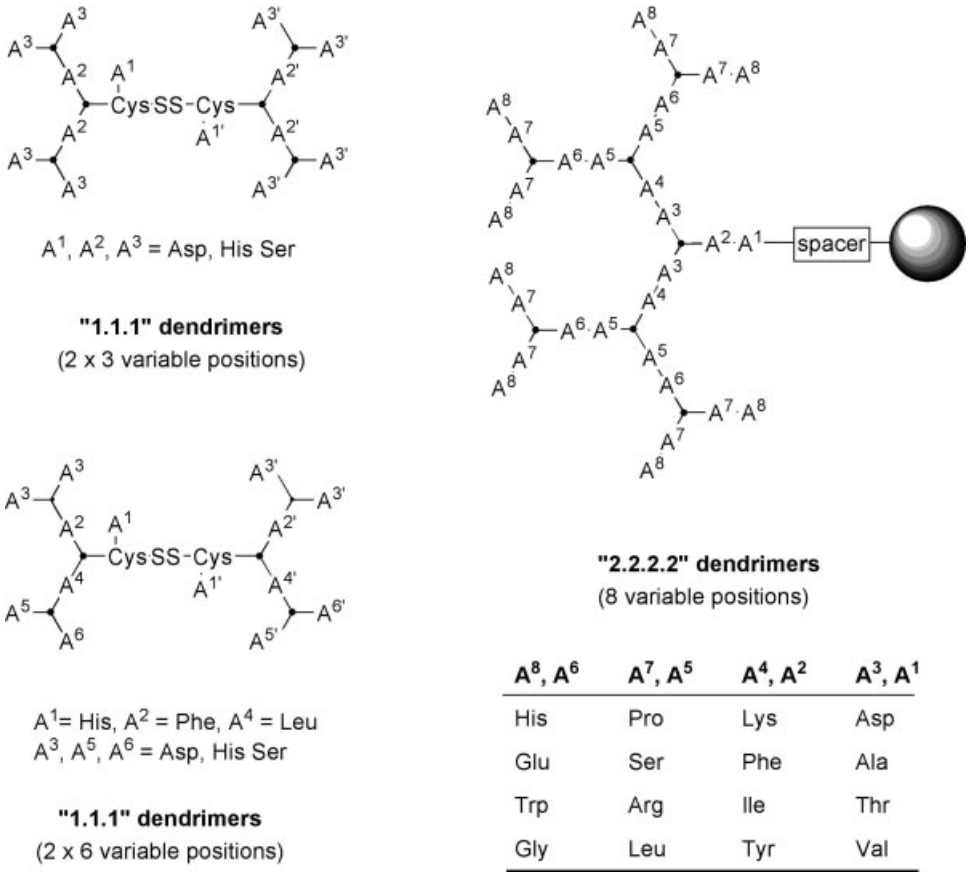
Design of the Peptide Dendrimer Combinatorial Library

Our initial studies of peptide dendrimers used a "1.1.1" topology with one variable amino acid per branch alternating with a diamino acid as branching unit (Figure 1).[2] Second-generation dendrimers were prepared and dimerized by disulfide-bond formation between a two-cysteine residue at the dendrimer core. This strategy allowed for six individually variable positions. Using an asymmetrically protected branching unit allowed for the definition of 6 individually addressable amino acid positions per dendrimer, corresponding to 12 variable positions in the disulfide-bridged dimer.[8] Although this approach did deliver esterase-like dendrimers from a small 27-member library built around a hydrophobic core and containing catalytic residues on the surface, the synthesis was relatively long and cumbersome.

In a separate series of experiments, we discovered that an alternative "2.2.2.2" topology incorporating two variable amino acids in each branch delivered third-generation dendrimers in yields of up to 35% after purification on HPLC.[9] This topology allowed for a total of eight variable amino acid positions in an overall 37 amino acid dendritic structure with a molecular weight of approximately 5 kDa. Since such dendrimers were accessible by direct synthesis on solid support without the need for convergent assembly in solution, we set out to develop a combinatorial approach to prepare combinatorial libraries of these "2.2.2.2" peptide dendrimers by the split-and-mix methodology.

Combinatorial split-and-mix peptide synthesis consists in a series of parallel solid-supported peptide syntheses in different reactors, whereby the solid-support samples undergoing functionalization in the different reactors are combined, mixed, and redistributed between the reactors after coupling of each amino acid.[6] This protocol allows the combinatorial realization of all possible amino acid combinations of the building blocks used. Most advantageously, each polymer bead carries peptides with only one of the possible sequences because it can only be present in one reactor for each of the coupling steps. Exhaustive coverage of sequences is possible only if the number of combinations in the library is smaller than the number of polymer beads used in the synthesis. We selected to use four different amino acids at each of the eight variable amino acid positions in the "2.2.2.2" dendrimer sequence, resulting in a $4^8 = 65{,}536$ member library, ensuring approximately 15-fold coverage for a synthesis using 0.4 g of resin containing approximately 1×10^6 polymer beads.

While split-and-mix combinatorial libraries are relatively easy to prepare, they can only be used for screening if the compound on each bead can be identified. The amount of product on a polymer bead (50–200 pmol) is sufficient for Edman sequencing or MS sequencing in the case of linear peptides. However these methods are relatively slow and expensive. Alternatives include binary encoding using tags,[10] but this doubles the number of synthetic operations because each tag must be coupled separately after each coupling step. In our dendrimer library approach we selected to take advantage of the dendritic architecture, which allowed us to design a self-encoded



A8, A6	A7, A5	A4, A2	A3, A1
His	Pro	Lys	Asp
Glu	Ser	Phe	Ala
Trp	Arg	Ile	Thr
Gly	Leu	Tyr	Val

FIGURE 1 Combinatorial library design in peptide dendrimers. "1.1.1" dendrimers were used to make small libraries of 27 different dendrimers using (1,3-diamino-2-propyl)oxy acetic acid or L-2,3-diamino-propanoic acid (Dap) as branching unit. "2.2.2.2" dendrimers are suitable for split-and-mix combinatorial libraries. The library shown realizes 65,536 (= 4^8) peptide dendrimers on tentagel solid support (grey sphere). L-2,3-Diamino-propanoic acid (Dap) was used as the branching unit. The N-terminus at position A^8 is acetylated.

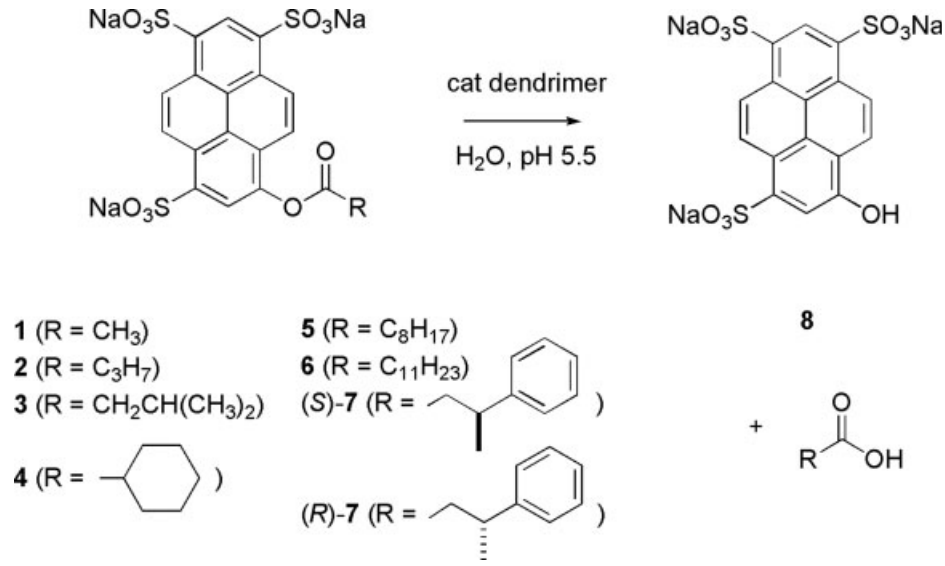
library decodable by simple amino acid composition analysis.

Amino acid composition analysis consists in total acidic hydrolysis of a given protein or peptide sample, derivatization of the free amino acids with a tag, usually PITC (phenyl isothiocyanate), and identification and quantification of each amino acid by an optimized HPLC analysis. Each amino acid would be utilized only twice during assembly of the combinatorial dendrimer library in two defined positions in successive branches (Figure 1). In this manner, any amino acid would occur with a relative abundance of 0, 1, 2, or 3, corresponding to either its absence (0) or its presence in the lower branch (1), the higher branch (2), or in both branches (3). The information from amino acid analysis would be redundant since the presence of any amino acid at a given position would be confirmed by the absence of the other amino acids at that position. Most advantageously, amino acid analysis is a routine procedure in biochemistry that can be operated automatically for a large number of samples at very low cost per sample.

The self-encoded library design described above leads to the distribution of 16 proteinogenic amino acids in four groups of four amino acids each. Amino acids were assigned to the variable positions with the aim of selecting esterase peptide dendrimers. Thus, histidine, a key catalytic residue, was placed in the fourth group present at the dendrimer surface in positions A^6 and A^8. Other amino acids were distributed evenly to allow for selection of optimal combinations.

Synthesis and Screening of the Peptide Dendrimer Combinatorial Library

Library synthesis was realized using Fmoc chemistry with benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate (BOP) in DMF as cou-



SCHEME 1 Peptide dendrimer catalyzed ester hydrolysis reactions.

pling reagent on a tentagel resin bearing the photolabile 4-(4-hydroxymethyl-2-methoxy-5-nitrophenoxy) butanoic acid at 0.28 mmol/g loading. The sequence was initiated by glycine and 6-aminohexanoic acid as spacer, followed by the first variable position. Dap was used as the branching unit. Synthesis was carried out in polypropylene syringes from which the resin beads could be easily washed out for the mixing operation. Double coupling and longer coupling times were necessary for completion toward the end of the synthesis, in particular after addition of the third branching diamino acid. The library was stored in Fmoc-protected form.

Small portions of the library (50 mg, ca. 100,000 beads) were treated with piperidine : DMF (1:4) for N-terminal Fmoc removal, acetylated, and the amino acid side chains were deprotected by treatment with the cleavage reagent trifluoroacetic acid (TFA) : 1,2-ethanedithiol (EDT) : H_2O : triisopropylsilane (TIS) 94:2:2:2. The beads were then washed several times with H_2O, methanol, and DMF and suspended in 1.5 mL of aqueous 20 mM Bis–Tris buffer, pH 6.0, containing 80 μM of the fluorogenic esterase substrate 8-butyryloxypyrene-1,3,6-trisulfonate (**2**) (Scheme 1). After 5 min of soaking, the beads were spread out on a petri dish. Within 5–10 min the excess buffer solution evaporated, leaving single beads on the dish surface without solvent between them. The beads retained sufficient solvent in them to allow the reaction to proceed. A green fluorescence appeared under UV 365 nm after 2 h in approximately 20% of the beads, indicating product formation, with few beads showing intense fluorescence (Figure 2). The intensely fluorescent beads were manually picked up using a pipette tip, transferred to an amino acid analysis glass vial, and submitted for amino acid analysis.

Ten beads showing strong fluorescence were analyzed by amino acid composition analysis as discussed above (Table I). In all cases the dendrimer sequence could be unambiguously assigned from the amino acid integration data. Six of 10 dendrimers contained histidine, known to be a catalytic residue, at position A^8 (60 versus 25% random) and 3 of these contained histidine at positions A^8 and A^6 (30 versus 6.1% random). The four other sequences did not contain any histidine residues. Sequencing of beads not selected for the fluorogenic reaction gave very different sequences with no overrepresentation of either

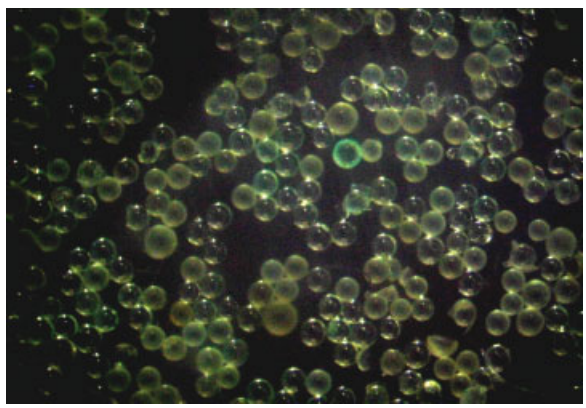


FIGURE 2 Screening of catalytic peptide dendrimer library. Microscope picture under illumination with UV 365 nm. Beads were soaked with 80 μM 8-butyryloxypyrene-1,3,6-trisulfonate **2** in aq 20 mM Bis–Tris, pH. 6.0, and spread out in a petri dish. The green bead near the center contains a catalytic sequence.

Table I Sequences of Hits D1–D10 Identified for Hydrolysis of Butyrate Easter 2 and Consensus Sequences D11/D12

Position[a]	AA	t_R (min)[b]	Integration[c]	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
A3, A1	Asp	1.88	9.9		5.8				5.7	7.3			23.5		
	Thr	4.54	4.1			17.8	23.9								
	Ala	4.75	10.2	10.8	7.4		13.8			32.3	26.4				
	Val	7.54	7.5	15.8		15.2		37.8	8.6		9.6	27.1	10.1		
A4, A2	Tyr	6.72	9.8		13.9	21.7									
	Ile	9.18	5.3	16.9	4.5	11.2	9.5	40.7	12.7		25.1		23.3		
	Phe	10.06	11.3							36.7		12.6			
	Lys	10.98	7.9	37.7			27.1					21.8			
A7, A5	Ser	3.25	10.7	50.3	32.6			73.2		37	106.1	140.3			
	Arg	4.22	8.9	36.2				101.1	67.7		55.4		123.8		
	Pro	5.08	13.8				250.2					75.4	69.4		
	Leu	9.36	11.6		79.8	163				32.1					
A8, A6	Glu	2.07	12.0					80.3							
	Gly	3.52	10.0	24.2		37.1	31.2	36.1	38.4		77.6		223.2		
	His	3.8	10.9		134.4	133.6	76.7			40.6	145.2	239.6			
	Trp	n.d.	0.0												
A8				W	**H**	**H**	**H**	**E**	G	**H**	**H**	**H**	G	**H**	**H**
A7				**S**	L	**L**	P	R	R	**S**	**S**	**S**	R	**S**	**S**
A6				G	**H**	**G**	**G**	**G**	W	**H**	**G**	**H**	G	**G**	**H**
A5				R	**S**	**L**	P	**S**	R	**L**	R	P	P	**S**	L
A4				**K**	Y	Y	**K**	**I**	**I**	F	I	**K**	**I**	**I**	**K**
A3				**V**	A	T	T	**V**	**V**	A	A	**V**	D	**V**	**V**
A2				**I**	**I**	I	**I**	**I**	**I**	F	**I**	F	**I**	**I**	**I**
A1				A	D	**V**	A	**V**	A	D	**V**	**V**	**V**	**V**	**V**
Isolated yield after resynthesis and HPLC purification (%)											12.8%		7.6%	8.4%	19.7%

The observed HPLC integration data are given in area counts, and the deduced amino acid sequence is shown in the lower part of the table.

[a] Amino acid position in dendrimer as shown in Figure 1.

[b] Retention time of PITC derivative in HPLC analysis;

[c] Reference integration value for amino acid.

histidine or basic amino acids, confirming that the sequences observed in fluorescent beads in the assay with the fluorogenic ester **2** reflected an activity selection and not a possible composition bias that might have occurred during synthesis. The amino acid composition analysis proved very reliable for sequence determination. Additional MS analysis of the dendrimers using photodeprotection of the photolabile linker was therefore not investigated.

Two of each type of hits, **D8** and **D10**, were resynthesized. Additionally, two consensus sequences **D11** and **D12** were prepared with the presumably catalytic diad His–Ser at A^8A^7; the first and second most frequent amino acid at A^6, A^5, and A^4; and the most frequently observed sequence Val–Ile–Val for $A^3A^2A^1$. Each of these four sequences was obtained in good yields (7–20% for 12 coupling steps), and their purity and identity was checked by HPLC and MS analysis.

Kinetic Studies of Catalytic Peptide Dendrimers

Kinetic studies were carried out to characterize the esterolytic activity of dendrimers **D8**, **D10**, **D11**, and **D12**. All kinetic studies were carried out in aqueous 20 m*M* citrate buffer, pH 5.5, which had been identified in another study as an optimal buffer system for peptide dendrimers.[9] Although the conditions are not very different from those in the Bis-Tris buffer, pH 6.0, used for screening, the lower pH results in a lower uncatalyzed background reaction. Catalysis was measured with 5 μM dendrimer as catalyst and 10–800 μM substrate. We used a series of structurally similar pyrene–trisulfonate esters **1–7**, including short, long, and branched aliphatic esters as well as (*R*)- and (*S*)-3-phenyl butyrate esters as chiral probes (Scheme 1).

The substrates were either commercially available (**1, 2,** and **5**, **6**) or were prepared by esterification of the sodium salt of hydroxypyene trisulfonic acid **8** with the corresponding acyl chloride and triethylamine at 0°C in DMF. The fluorogenic substrates were obtained in almost quantitative yield after preparative reverse-phase HPLC purification (**3**, **4**, (*R*)-**7**, (*S*)-**7**). All substrates were diluted from freshly prepared 10 m*M* stock solutions in acetonitrile : water 1:1. Reactions were run in 25 μL volume in half-area 96-well polystyrene plates and followed using a fluorescence microtiter plate reader.

Initial studies with the butyrate ester **2** showed that sequence **D10**, which did not contain a histidine residue, was not catalytically active. This observation is in agreement with the fact that all previously studied esterase peptide dendrimers required histidine for catalytic activity with these substrates and suggests that the four hits from the library not containing histidine represent false positives in the screening process. These hit sequences all contained arginine at positions A^5/A^7, suggesting that they might strongly bind and accumulate substrate during the library equilibration process by electrostatic interactions, leading to a higher fluorescence signal from background hydrolysis without specific catalysis.

All three histidine-containing dendrimers **D8**, **D11**, and **D12** showed enzyme-like catalysis with substrate **2**, with substrate binding $K_M \sim 0.1$ m*M*, catalytic rate constant $k_{cat} \sim 0.1$ min^{-1}, and specific rate acceleration $k_{cat}/k_{uncat} \sim$ 2,000–10,000 (Table II, Figure 3). Catalysis was proportional to dendrimer concentration and showed multiple turnovers. There was no detectable product inhibition or catalyst inactivation upon reaction, as evidenced by the fact that addition of fresh substrate after completion of a dendrimer-catalyzed reaction resulted in further hydrolysis of substrate **2** at the same initial rate as in the first reaction.

Compared to catalysis with the small molecule catalyst 4-methyl-imidazole (4-MeIm), as a model for the histidine side chain, the dendrimers were up to 2,200× more active. Assuming that each histidine side chain is catalytically active, this translates into an approximately 270-fold enhancement per histidine residue. These three catalytic peptide dendrimers possess a hydrophobic core (Val, Ile, or Ala at $A^3.A^2A^1$, the same His–Ser sequence at the outermost layer A^8A^7, and differ mostly at positions $A^6A^5.A^4$ (**D8**: GR.I; **D11**: GS.I; **D12**: HL.K). Dendrimer **D11** was much less active than either **D8** or **D12**, suggesting a critical role of their additional basic residues (4 × Arg at A^5 for **D8**, 4 × His at A^6, and 2 × Lys at A^4 for **D12**) in catalysis. These additional basic residues might enhance catalysis by electrostatic transition state stabilization or by lowering of the p*K*a of the catalytic histidine residues at A^8.

Efficient catalysis was also observed with the acetate ester **1** and the nonanoate ester **5**. The branched substrates **3** and **4** however showed much lower catalytic efficiencies, with rate acceleration in the range of $k_{cat}/k_{uncat} \sim$ 500–1000. The lower catalytic activtiy of the dendrimers with the branched substrates **3**, **4**, and **7** parallels the second-order rate constants measured with 4-MeIm, a small molecule analog of the histidine side chain. A lower reactivity of these substrates would be compatible with direct nucleophilic catalysis by the imidazole, in which steric hindrance by the acyl chain might influence the reaction rate. The putative acyl dendrimer intermediate could however not be detected, which would imply that this intermediate is hydrolyzed faster than it is formed.

Table II Kinetic Parameters for Esterolytic Peptide Dendrimers

		1	**2**	**3**	**4**	**5**	(*S*)-**7**	(*R*)-**7**
	k_{uncat} (min^{-1})	2.9×10^{-5}	1.5×10^{-5}	5.2×10^{-5}	6.5×10^{-5}	1.0×10^{-5}	3.8×10^{-5}	
	k_2 (4-Melm) (mM^{-1}) min^{-1})	1.1×10^{-3}	3.9×10^{-4}	1.0×10^{-4}	3.3×10^{-4}	7.2×10^{-4}	1.1×10^{-4}	
D8	K_M (m*M*)	0.13	0.21	0.15	0.087	0.13	0.11	0.062
	k_{cat} (min^{-1})	0.17	0.13	0.012	0.035	0.052	0.022	0.016
	k_{cat}/k_{uncat}	5800	10000	240	540	5200	580	420
	k_{cat}/K_M	1.30	0.59	0.090	0.40	0.39	0.21	0.25
	$(k_{cat}/k_M)/k_2$	1100	1500	890	1200	550	1800	2100
	V_{net}/V_{uncat}	64	82	5.0	10	91	11	10
D11	K_M (m*M*)	0.17	0.12	0.25	0.21	0.019	0.024	0.010
	k_{cat} (min^{-1})	0.045	0.025	0.011	0.035	0.012	0.0057	0.0026
	k_{cat}/k_{uncat}	1600	2000	200	550	1200	150	68
	k_{cat}/K_M	0.26	0.22	0.040	0.17	0.64	0.24	0.26
	$(k_{cat}/K_M)/k_2$	220	560	400	510	890	2100	2200
	V_{net}/V_{uncat}	16	27	3.0	8.0	32	3.0	2.0
D12	K_M (m*M*)	0.25	0.15	0.11	0.12	0.11	0.30	0.14
	k_{cat} (min^{-1})	0.26	0.13	0.021	0.086	0.066	0.044	0.034
	k_{cat}/k_{uncat}	8800	10000	410	1300	6600	1200	890
	k_{cat}/K_M	1.0	0.85	0.19	0.74	0.60	0.15	0.25
	$(k_{cat}/K_M)/k_2$	840	2200	1900	2200	840	1300	2100
	V_{net}/V_{uncat}	91	102	9.0	18	98	15	15

Conditions: 10–800 μM substrate, 5 μM dendrimer. 20 m*M* aq citrate pH 5.5, 25°C. The kinetic constants given are derived from the linear double-reciprocal plots of $1/V_{net}$ versus $1/[S]$. V_{net}/V_{uncat} is the apparent rate enhancement observed with $S = 200$ μM and 2.5 mol% catalytic peptide dendrimer (5 μM). $V_{net} = V_{app} - V_{uncat}$ with V_{app} being the apparent hydrolysis rate in the presence of dendrimer and V_{uncat} the hydrolysis rate in buffer alone. $k_2(I_m)$ is the second-order rate constant for 4-MeIm catalysis measured under the same conditions.

The branched substrates **3**, **4**, (*S*)-**7**, and (*R*)-**7** showed a higher background hydrolysis rate in the absence of dendrimer compared to **1**, **2**, and **5** with linear acyl chains. The same instability was observed with dodecanoate **7**, which did not show any catalysis with the dendrimers. This unusual reactivity profile is unexplained at this point and might result from the formation of stabilizing or destabilizing aggregates of the substrates. This uncatalyzed reaction was directly proportional to substrate concentration in all cases.

By contrast to the large differences in the catalytic rate constant k_{cat}, all three dendrimers showed comparable substrate binding K_M across the different substrates despite of the large variations in hydrophobicity of the acyl chains and the presence of additional positively charged residues in **D8** and **D12** compared to **D11**. This situation has been observed previously in our "1.1.1" peptide dendrimers with a hydrophobic core, where only one of four catalytic dendrimers showed increased substrate binding as a function of substrate hydrophobicity in the case of the nonanoyl ester **5** compared to butyrate **2** and acetate **1**.[8] Also, no significant chiral discrimination was observed between the enantiomeric substrates (*R*)/(*S*)-**7**. These data suggest that the mere presence of hydrophobic amino acids at the core is not sufficient to induce differentiating hydrophobic contacts with the substrates.

CONCLUSION

A combinatorial library approach to catalytic peptide dendrimers has been demonstrated. The "2.2.2.2" topology chosen allows for the formation of third-generation peptide dendrimers in excellent yields across a broad range of sequences. Structural diversity is installed by combinatorial variations of eight variable amino acid positions within the core, branches, and at the surface. The self-encoded nature of the dendrimer libraries, which allows sequence determination by simple amino acid analysis, is particularly attractive because the analysis is rapid, inexpensive, reliable, and does not require additional synthetic operations for library tagging during synthesis.

The direct screening procedure for catalysis used here is noteworthy and has not been reported previously. Our assay consists in simply soaking the polymer beads with a fluorogenic substrate and spreading them out on a dry surface to prevent diffusion of the soluble product formed, which allows the beads containing active dendrimers to be visible. This method

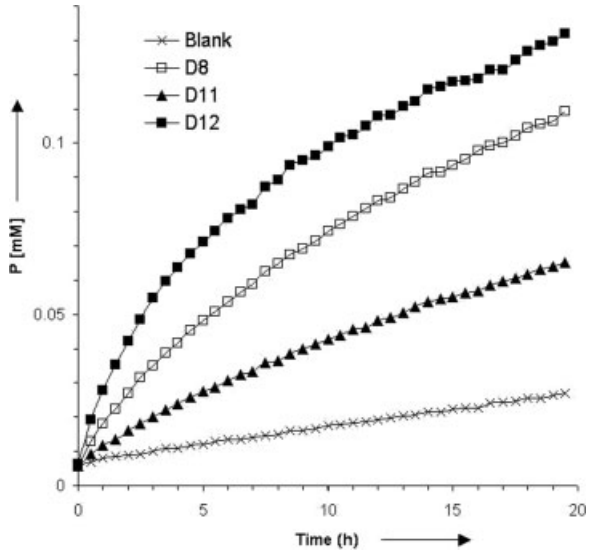


FIGURE 3 Hydrolysis of 8-butyryloxypyrene-1,3,6-trisulfonate **2** catalyzed by peptide dendrimers from the combinatorial library. Conditions: 1000 μM substrate, 2.5 μM dendrimer (0.25 mol %), aq 20 m*M* citrate, pH 5.5, 25°C. Fluorescence was converted to product concentration using a calibration curve, which was linear in the concentration range used.

circumvents the problem of detecting catalysis on beads while preventing product diffusion and should be generally applicable for all chromogenic and fluorogenic detection systems, typically those used for enzyme assays.[11] Direct on bead screening for catalysis in combinatorial catalyst libraries has also been reported using substrates forming insoluble precipitates as products[12] and with beads covalently modified with pH indicators[13] or reactive substrates.[14]

The combinatorial approach reported here allows one to systematically survey a very large number of peptide dendrimers for catalysis in a very efficient way. Beyond the proof of concept study discussed here, the system should prove useful for other, potentially more difficult problems in catalysis. Experiments are underway toward the discovery of enantioselective esterases by direct screening of dendrimer libraries using chiral substrates. Biased libraries containing catalytic residues at the dendrimer core are also under study to create an enzyme model with single catalytic sites.

MATERIALS AND METHODS

Analytical HPLC columns: Chromolith Performance RP-18e, 0.46 × 10 cm, flow 3 mL.min^{-1}; detection by UV at 214 nm, solvent systems: A = 0.1% TFA in H_2O; B = 40% H_2O / 60% CH_3CN, 0.1% TFA. Preparative RP–HPLC was performed with HPLC-grade acetonitrile and MilliQ deionized water in a Waters prepak cartridge 500 g (RP-C18 20 mm, 300 Å pore size) installed on a Waters Prep LC4000 (Rupperswil, Switzerland) system from Millipore (flow rate 100 mL·min^{-1}, gradient 1%·min^{-1} CH_3CN). HPLC conditions for dendrimer 8,10,C11,C12: A/B = 85/15 to A/B = 60/40 in 35 min. BOP and Fmoc–Cys(Trt)–OH were purchased from Novabiochem (Bleicherstrasse 11, Lucerne, Switzerland); Fmoc–His(1-Boc)–OH, Fmoc–Gly–OH, Fmoc–Dap(Fmoc)–OH were purchased from Bachem (Bubendorf, Switzerland); Fmoc–Ala–OH, Fmoc–Ile–OH, Fmoc–Leu–OH, Fmoc–Val–OH, Fmoc–Pro-OH, Fmoc–Phe–OH, Fmoc–Arg (Pbf)–OH, Fmoc–Trp(Boc)–OH, Fmoc–Lys(Boc)–OH, Fmoc–Asp(O*t*Bu)–OH, Fmoc–Glu(O*t*Bu)–OH, Fmoc–Tyr(*t*Bu)–OH, Fmoc–Thr(*t*Bu)–OH, Fmoc–Ser(*t*Bu)–OH were purchased from Senn Chemicals (Dielsdorf, Switzerland); *N*-ethyldiisopropylamine (DIEA) was purchased from Fluka (Buchs, Switzerland).

8-Isovaleryloxypyrene-1,3,6-trisulfonate (3). Isovaleryl chloride (34.85 μL; 0.286 mmol; 3 equiv) was added to a stirred solution of **8** (50 mg; 0.095 mmol) with triethylamine (39.84 μL; 0.286 mmol; 3 equiv) in DMF at 0°C. The reaction, which was followed by analytical HPLC, was complete in 2 h. After solvent evaporation, the residue was purified by preparative RP-HPLC (A/B = 90/10 to A/B = 60/40 in 30 min) and the product **3** was obtained as a yellow solid (56.2 mg; 97%); ^{1}H-NMR (D_2O): δ = 9.14–9.01 (m, 4H), 8.37 (s, 1H), 8.29 (d, J = 9.6 Hz, 1H), 2.66 (d, J = 7.3 Hz, 2H), 2.24–2.15 (m, 1H), 1.02 (d, 6H); ^{13}C-NMR (D_2O): δ = 175.2, 144.23, 138.37, 136.22, 129.57, 128.91, 127.11, 126.46, 125.41, 125.26, 124.83, 124.69, 123.20, 120.11, 42.58, 25.42, 21.62; HR ESI MS negative mode: calcd for $C_{21}H_{17}O_{11}S_3$; 540.9776; found, 540.9993.

8-Cyclohexanecarboxyloxypyrene-1,3,6-trisulfonate (4). Cyclohexane carboxyl chloride (33.90 μL; 0.286 mmol; 3 equiv) was added to a stirred solution of **8** (50 mg; 0.095 mmol) with triethylamine (39.84 μL; 0.286 mmol; 3 equiv) in DMF at 0°C. The reaction, which was followed by analytical HPLC, was complete in 2 h. After solvent evaporation, the residue was purified by preparative RP-HPLC (A/B = 90/10 to A/B = 60/40 in 30 min) and the product **4** was obtained as a yellow solid (57.4 mg; 95%); ^{1}H-NMR (D_2O): δ = 9.15–8.98 (m, 4H), 8.29 (s, 1H), 8.13 (d, J = 9.6 Hz, 1H), 2.57 (m, 1H), 1.98–1.95 (m, 2H), 1.69–1.46 (m, 6H), 1.24–1.14 (m, 2H); ^{13}C-NMR (D_2O): δ =162.21, 144.51, 138.42, 136.22, 136.12, 129.60, 128.96, 127.11, 126.44, 125.51, 125.27, 124.87, 124.65, 123.16, 120.16, 42.99, 28.66, 25.23, 24.91; HR ESI MS negative mode: calcd for $C_{23}H_{19}O_{11}S_3$; 566.9933; found, 567.0906.

(R)-8-Phenybutyryloxypyrene-1,3,6-trisulfonate (7). To a solution of (*R*)-3-phenylbutyric acid (ee $\geq$ 99%) (87.8 μL; 0.57 mmol) in dry DCM was added a catalytic amount of dry DMF (2 drops), with triethylamine (87.5 μL; 0.63 mmol, 1.1 equiv) and oxalyl chloride (59.1 μL; 0.57 mmol, 1.1 equiv) under nitrogen. The reaction was stirred 2 h at room temperature and then the mixture solution was con-

centrated at 600 mmHg for 10 min to remove DCM and excess of oxalyl chloride. A solution of **8** (100 mg; 0.19 mmol, 0.34 equiv) with triethylamine (79.5 μL; 0.57 mmol; 3 equiv) in DMF at 0°C was added to the concentrated solution of acyl chloride. The reaction, which was followed by analytical HPLC, was complete in 5 h. After solvent evaporation, the residue was purified by preparative RP-HPLC (A/B = 90/10 to A/B = 50/50 in 25 min) and the product (*R*)-**7** was obtained as a yellow solid (119.6 mg; 94%); ^{1}H-NMR (D_2O): δ = 9.20–9.11 (m, 3H), 8.76 (d, J = 9.6 Hz, 1H), 8.06 (s, 1H), 7.37–7.26 (m, 3H), 7.01 (d, J = 7.7 Hz, 2H), 6.82 (d, J = 9.6 Hz, 1H), 3.05–2.88 (m, 2H), 2.61–2.52 (m, 1H), 1.19 (s, 3H); ^{13}C-NMR (D_2O): δ = 176.72, 147.16, 146.38, 140.86, 138.68, 131.56, 129.80, 129.57, 129.48, 128.99, 127.78, 127.42, 127.05, 126.99, 125.15, 122.42, 44.67, 39,44, 24.10; HR ESI MS (negative mode): calcd for $C_{26}H_{17}O_{11}S_3$; 600.9919; found, 600.9933.

(S)-8-Phenybutyryloxypyrene-1,3,6-trisulfonate (7). Starting with (*S*)-3-phenylbutyric acid (ee $\geq$ 99%) and following the same procedure described for the compound (*R*)-**7**, the product (*S*)-**7** was obtained as a yellow solid (120.87 mg; 95%). HR ESI MS negative mode: calcd for $C_{26}H_{17}O_{11}S_3$; 600.9919; found, 600.9857.

Synthesis of the Library of On-Bead Dendrimers by SPPS

Coupling of the Fmoc-Protected Amino Acids. The resin was washed and swelled inside the reactor with DCM (2 × 5 mL) and DMF (1 × 5 mL). The Tentagel resin (0.23 mmol/g) was acylated with 2.5 equivalents of *N*-Fmoc amino acid in the presence of 2.5 equiv of BOP and 6 equiv of DIEA in DMF. After 30 min (first generation), 1 h (second generation), and 2 h (third generation) the resin was washed (3× each) with DMF, dichloromethane (DCM), and MeOH and controlled with the trinitrobenzenesulfonic acid (TNBS) test.

Cleavage of Fmoc-Protecting Group. The Fmoc-protecting group was removed with 5 mL of a solution of piperidine in DMF (1:4) for 10 min. After filtration, the procedure was repeated and then washed (3× each) with DMF, DCM, and MeOH.

Capping of the N-Terminus. At the end of the synthesis, the resin was acetylated with a solution of acetic anhydride in DCM (1:1) for 30 min and washed with DCM, DMF, and methanol. The resin was dried under vacuum and stored at −20°C.

TFA Cleavage of Protecting Group. The cleavage was carried out using TFA : EDT : H_2O : TIS 94:2:2:2 solution for 3 h. The library then was washed with H_2O, methanol, and DMF and dried under vacuum and stored at −20°C.

Dendrimer D8. From Tentagel Rink amide resin (160 mg, 0.25 mmol/g), the dendrimer **D8** was obtained as a colorless foamy solid after preparative HPLC purification (6.4 mg, 12.8%); RP-HPLC: t_R = 22.4 min; MS (ES+): calcd for $C_{178}H_{283}N_{75}O_{55}$: 4351.16, found: 4352.50.

Dendrimer D10. From Tentagel Rink amide resin (160 mg, 0.25 mmol/g), the dendrimer **D10** was obtained as a colorless foamy solid after preparative HPLC purification (3.8 mg, 7.6%); RP-HPLC: t_R = 23.8 min; MS (ES+): calcd for $C_{170}H_{295}N_{71}O_{47}$: 4083.28, found: 4085.63.

Dendrimer D11. From Tentagel Rink amide resin (160 mg, 0.25 mmol/g), the dendrimer **D11** was obtained as a colorless foamy solid after preparative HPLC purification (4.2 mg, 8.4%); RP-HPLC: t_R = 22.5 min; MS (ES+): calcd for $C_{170}H_{273}N_{63}O_{59}$: 4130.95, found: 4132.13.

Dendrimer D12. From Tentagel Rink amide resin (160 mg, 0.25 mmol/g), the dendrimer **D12** was obtained as a colorless foamy solid after preparative HPLC purification (19.7 mg, 39.4%); RP-HPLC: t_R = 21.9 min; MS (ES+): calcd for $C_{199}H_{305}N_{71}O_{56}$: 4585.32, found: 4586.88.

Kinetic Measurements. The kinetic measurements were carried out by using a SPECTRAMax fluorescence detector (Bucher Biotec, AG, Basel, Switzerland) with preset values of the excitation and emission wavelengths corresponding to the measured pyrene substrate (λ_{ex} = 460 nmμ, λ_{em} = 530 nm) at 25°C. Assays were followed in individual wells of round-bottom polystyrene 96-well plates (Costar) (Life Technologies, Uferstrasse 90, Basel, Switzerland). Kinetic experiments were followed for 2 h. The dendrimers were stored at −20°C in 1 m*M* stock solution in acetonitrile : water: 1/1. Dendrimer stock solutions were freshly diluted to 0.05 m*M* solution in 20 m*M* aq citrate, pH 5.5. The citrate buffer, pH 5.5, was prepared using MilliQ deionized water. Fluorescence data were converted to product concentration by means of a calibration curve. Initial reaction rates were calculated from the steepest part observed during the first 2000 s of each curve. In a typical experiment, 20 μL of aq citrate, pH 5.5 (20 m*M*), were first added in a well and then 2.5 μL of a dendrimer solution (0.05 mM in aq.citrate pH, 5.5, final concentration in the well: 5 μM), and finally 2.5 μL of substrate solution (2 m*M* in acetonitrile : water 1:1, final concentration in the well: 5 μM). The rate observed under these conditions is the apparent rate V_{app}. V_{uncat} is the rate observed with 22.5 μL aq citrate, pH 5.5 (20 m*M*), and 2.5 μL of substrate solution (2 m*M* in acetonitrile : water 1:1, final concentration in the well: 200 μM). The observed rate enhancement V_{net}/V_{uncat} is defined as $(V_{app}/V_{uncat}) - 1$.

Michaelis–Menten parameters were obtained from the linear double reciprocal plot of $1/V_{net}$ versus $1/[S]$ measured similarly with (final concentrations) 5 μM dendrimer (V_{app}) or no dendrimer (V_{uncat}) and 10, 20, 40, 60, 80, 100, 200, 400, 600, 800, 1000 μM substrate, 20 m*M* citrate pH 5.5, 25°C. Kinetic parameters such as maximum velocity (V_{max}) and Michaelis constant (K_M) were determined by the least squares method from a Lineweaver–Burk plot between $1/v_0$ (reciprocal value of initial velocity) and $1/[S]$. The reaction

activity reflected in k_{cat} for the hydrolysis (deacylation of substrates) was evaluated by $k_{cat} = V_{max}/[D]_0$, where $[D]_0$ indicates the initial concentration of dendrimers. The reaction rate with 4-MeIm was obtained under the same conditions with 50, 100, 200, 400, 600, 800, 1000, and 1500 μM 4-MeIm and 200 μM substrate. The second-order rate constants k_2 were calculated from linear regression of the experimentally measured pseudo-first-order rate constants k' as a function of 4-MeIm concentrations $[M]$ where $[S]$ indicates the initial substrate concentration: $V_{net} = k'[S]$, $k' = k_2[M]$.

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