Identification of Catalytic Peptide Dendrimers by "Off-Bead" in Silica High-Throughput Screening of Combinatorial Libraries

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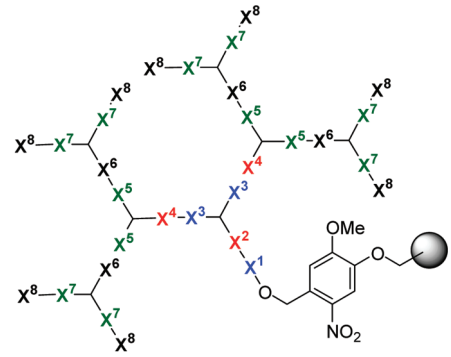
A combinatorial library of up to 65′536 peptide dendrimers $(AcX^8X^7)_8(DapX^6X^5)_4(DapX^4X^3)_2DapX^2X^1$ (Dap = (*S*)-2,3-diaminopropionic acid branching point, X^{8-1} = groups of four proteinogenic L-amino acids) was prepared on a photocleavable tentagel resin. The library was assayed for catalytic hydrolysis of the fluorogenic substrate 1-butyryloxy-pyrene-2,7,8-trisulfonate **1** and analogs by a simple procedure involving (a) photocleavage from the support in the absence of solvent, (b) spreading of the solid support beads on the surface of a silicagel plate impregnated with an aqueous buffered substrate solution, and (c) identification of hits as beads surrounded by a fluorescent halo indicative of catalysis and sequence determination in hits and nonhits by amino acid analysis of the beads. The experiment provides direct access to structure−activity relationships in the library and delivers active esterase dendrimers. Anionic glutamate residues in the outer dendrimer branches were found to inhibit catalysis by histidine residues at the dendrimer core. The "off-bead" in silica assay is simple to implement and transferable to other library and reaction types.

Introduction

Split-and-mix synthesis on solid support as originally reported for peptides is one of the simplest and most efficient protocols in combinatorial chemistry.[1−5] However activity screening[6] and decoding[7] of the resulting "one-bead-one-compound" (OBOC)[8] libraries poses significant challenges and problems. Indeed, "on-bead" activity assays, in which the library is tested by a staining reaction, while the compounds are attached on the synthesis beads, are simple to carry out but are often not predictive of the activity of the compound as a free molecules in solution.[9] Herein we report a simple and practical "off-bead" screening protocol for catalysis that allows one to assay OBOC libraries as free molecules in solution on the surface of silica gel plates. Its use is demonstrated for the identification of peptide dendrimers catalyzing the hydrolysis of acyloxypyrene trisulfonates **1**−**4** in library **L** of up to 65′536 third generation peptide dendrimers (Figure 1). Structure−activity relationships are revealed from the sequences of active and inactive beads. Investigations of resynthesized dendrimers in solution extend a previous study realized using an on-bead assay with the same library.[10]

Dendrimers are regularly branched synthetic macromolecules that can be assembled from a variety of building blocks and display a broad range of properties.[11,12] Recently we prepared combinatorial libraries of peptide dendrimers[13] by split-and-mix synthesis on solid-support to explore their functional potential as synthetic protein mimics.[14,15] The dendrimer sequences could be determined by quantitative amino acid analysis (AAA) of the beads thanks to a simple



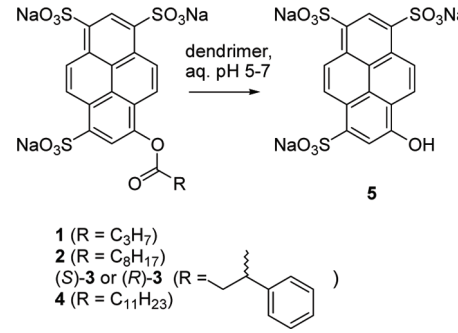


Figure 1. Structure of peptide dendrimer combinatorial library **L** for "off-bead" in silica HTS and fluorogenic ester substrates. X^{1-8} are the indicated amino acids, the C-terminus is at the core esterified to the photolabile linker. The branching points are (*S*)-2,3-diaminopropionic acid, and the 8 N-termini are acetylated.

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design algorithm.[16] AAA is realized by acidic hydrolysis, derivatization of the free amino acids to phenyl thiocarbamates, and separation by HPLC. Because AAA is a standard, inexpensive automated analysis, the sequence determination of 20−50 beads per assay was affordable, which greatly facilitated library screening. The method was also particularly attractive because no additional tags were necessary, and AAA provided a global composition analysis of the bead as a quality control measure, allowing incomplete sequences and empty beads to be discarded. A modified library design algorithm allowed us to extend this simple decoding method to linear and cyclic peptides.[17]

Library decoding by AAA greatly simplified library synthesis and sequence determination and allowed us to screen a variety of processes on beads. While binding assays, such as selection of ligands for Vitamin B_{12}[18] or for lectins,[19] gave good results, activity screening for catalysis turned out to pose significant problems. Our initial on-bead assay involved soaking the synthesis beads with a fluorogenic substrate and picking strongly fluorescent beads as positive hits.[16] Although many catalysts were properly identified by the assay, the method also produced artifacts, in particular positive hits for the hydrolysis of ester **1** in library **L** above lacking a histidine residue and showing no activity in solution after resynthesis. In addition, contaminating background fluorescence of the synthesis beads rendered picking of negative hits impossible, precluding a direct structure−activity relationship study by library screening.

To circumvent the limitations of the on-bead assay and further explore the structure−activity relationship in esterase peptide dendrimers from library **L**, we have developed an assay in which the library members can be tested after cleavage from the solid support. The method is related to the recently reported photocleavage protocol of Imperiali and co-workers to detect fluorescent terbium chelators, in which a peptide library immobilized as a suspension in agar was subjected to photocleavage to screen for fluorescence in the surrounding solution.[20] Our "off-bead" assay takes place at the surface of silica gel plates rather than in an agar suspension. Using this "off-bead" assay, we show that catalysis of ester hydrolysis in the core−active site peptide dendrimers from library **L** not only depends on the presence of histidine residues at the dendritic core but is also controlled by the outer dendritic branches, whereby anionic residues in these branches inhibit catalysis.

Results and Discussion

Development of the Off-Bead in Silica Assay. Assaying an OBOC library off-bead requires three operations: (1) spatial isolation of the beads, (2) cleavage of the compounds from the solid support into the surrounding medium, and (3) identification of active compounds by an activity assay in the medium surrounding the beads and determination of the active library member by decoding. Spatial isolation of the beads has been reported by robotic arraying into microtiter plates.[21] However, this approach is instrumentally heavy and much more difficult to realize than a simple on-bead assay. We therefore turned to a direct cleavage assay similar to the Imperiali protocol,[20] in which library beads are isolated spatially by immobilization in an agarose matrix, and the peptides are released by photolysis from a photolabile linker.

Initial experiments were conducted with the first generation dendrimer **RG0** $(AcArgSer)_2DapHisSerOH$, and its noncatalytic alanine mutant **RG0A** $(AcArgSer)_2DapAlaSerOH$ synthesized on a photolabile resin (Figure 2B). Dendrimer **RG0** corresponds to the catalytic core of dendrimer **RG3** $((AcTyrThr)_8(DapTrpGly)_4(DapArgSer)_2DapHisSerNH_2)$.[10] The pair of arginine residues in the first generation branch mediate binding of substrate **1** by electrostatic interactions with the sulfonate groups, and the histidine residue at the core triggers ester hydrolysis by general base or nucleophilic catalysis. While **RG0** exhibits moderate ester hydrolysis activity, its alanine mutant **RG0A** is expected to bind to substrate **1** but cannot catalyze its hydrolysis because it lacks the critical histidine residue. Both dendrimers were prepared on a hydroxymethyl-photolinker NovaSynTG resin (0.24 mmol g^{-1}), and the side-chains were deprotected by acidic treatment.

Photolysis of an agarose suspension of the dendrimer-functionalized tentagel beads after solidification in a Petri dish as described by Imperiali et al.,[20] followed by addition of an aqueous solution of substrate **1** on the agarose surface, did not provide any observable fluorescence signal in the suspension. The absence of a detectable signal might be caused by a rapid diffusion, and presumably dilution, of the dendrimers into the surrounding agarose either during photolysis or after the addition of the substrate.

To limit the possibility of dilution effects, we envisioned a different procedure in which the dendrimers would be photolyzed from the support in the absence of solvent. The photolyzed dried beads would then be spread out on the surface of a silica gel plate previously impregnated with a solution of the fluorogenic substrate to be tested. We have shown previously that this in silica format is suitable for a low-volume high-throughput screening of enzyme activities.[22] In the present case, contact of a dry, dendrimer-loaded synthesis bead with the wet silica gel surface would induce solubilization of part of the dendrimer and its diffusion into the surrounding matrix where a fluorogenic reaction might take place and be detectable visually.

To carry out a solvent free photolysis, the polymer beads were equilibrated with the assay buffer, dried by filtration under vacuum, dispersed in a Petri dish, and subjected to UV irradiation with a 100 W mercury lamp during 30 min. Photolysis with 100 mg of solid-supported **RG0** under these conditions, followed by washing with water, provided the pure dendrimer in 25% yield, which is comparable to the yields obtained from an acidic cleavable carrier. For high-throughput screening, the photolyzed dried beads containing **RG0** were put in direct contact with silica gel plate freshly impregnated with a solution of substrate **1** in buffer (Figure 2A). After 1.5 h at 25 °C, fluorescent halos around the beads were clearly visible under irradiation with a 366 nm UV lamp, suggesting that the dendrimer was diffusing into the silica gel plate (Figure 2C). By contrast, the same experiment carried out with the control, noncatalytic dendrimer **RG0A** did not result in any detectable fluorescent halo around the

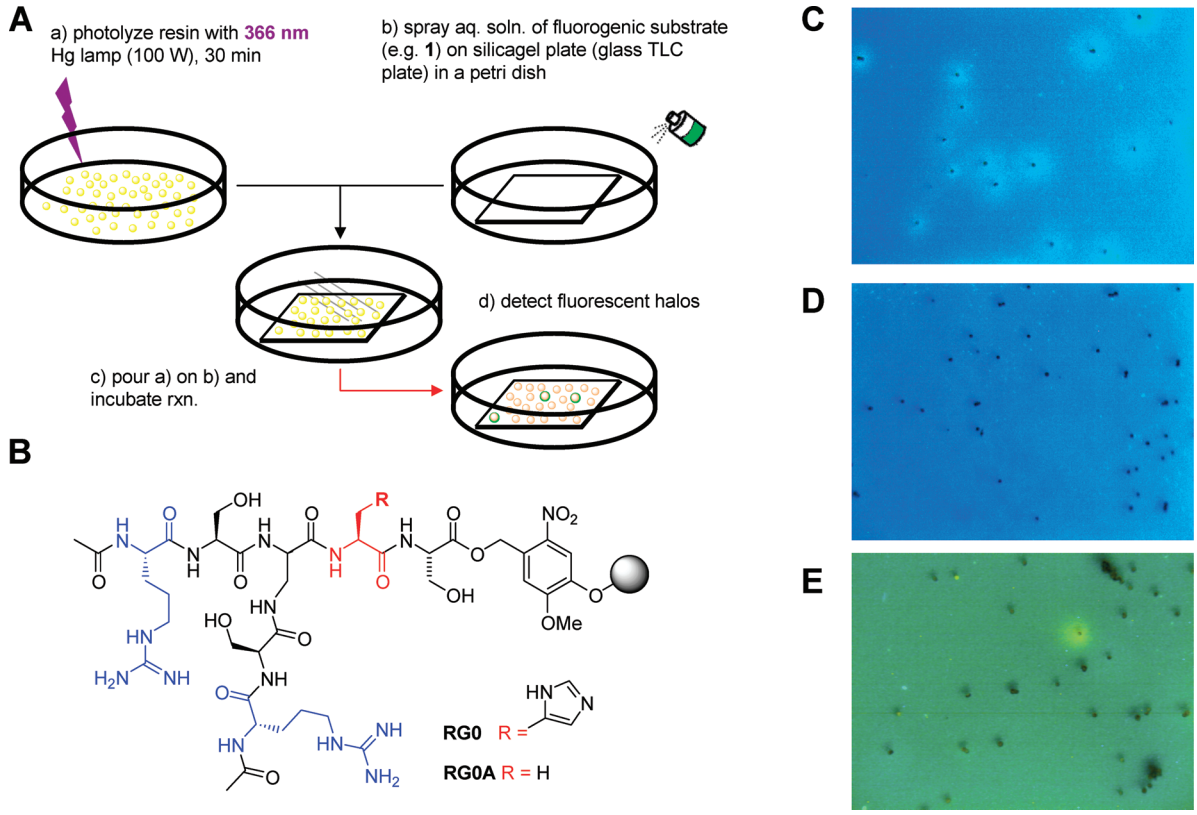

Figure 2. A. Principle of off-bead in silica screening. B. Structure of control active (**RG0**) and inactive (**RG0A**) dendrimers. C. Photograph of a silica gel plate impregnated with a 80 μM **1** in bis-tris buffer pH 6.0 in contact with photolyzed beads carrying **RG0** $(AcArgSer)_2DapHisSerOH$. **D**. Same as B with photolyzed beads carrying **RG0A** $(AcArgSer)_2$*Dap*AlaSerOH. E. Off-bead HTS assay with library **L**.

beads (Figure 2D). AAA of the beads picked from the silica gel surface after the catalysis assay showed the expected amino acids in levels sufficient for sequence determination. The retention of significant amounts of dendrimers within the beads might be caused either by partial photolysis or by limited diffusion of the cleaved dendrimer from the bead during the assay.

off-Bead in Silica High-Throughput Screening. The procedure was next applied to screen library **L** for esterolytic activity against acyloxypyrene-trisulfonates **1**−**3**, which were either commercially available (**1**−**2**), or were prepared from hydroxypyrene trisulfonate **5** and (*R*)- or (*S*)-3-phenylbutyric acid ((*S*)-**3** and (*R*)-**3**). While we had reported a detailed study of two esterolytic dendrimers from this library featuring a catalytic core surrounded by hydrophobic residues,[10] a more extended structure−activity relationship study implicating the combinatorial library itself had not been carried out because of the limitations of the on-bead assay.

Library **L** was prepared by split-and-mix synthesis on a 500 mg batch of hydroxymethyl-photolinker NovaSyn TG resin (0.24 mmol g^{-1}). Library synthesis was completed by treatment with piperidine to remove the last Fmoc protecting group, acetylation of the N-terminus, and removal of the side-chain protecting groups by acidic treatment. The quality of the library was checked by submitting 27 beads to amino acid analysis. Twenty beads (75%) returned a readable sequence, showing the expected even distribution of amino acids at each position (Tables S1 and S2, Supporting Information). The remaining contained an incomplete sequence, reflecting the yield of library synthesis.

In a typical catalysis HTS assay, 10 mg of resin were equilibrated with aqueous 20 mM bis-tris buffer pH 6.0, dried by filtration under vacuum, spread out on a 3 cm Petri dish, and irradiated with a 100 W mercury lamp for 30 min. The photolyzed resin beads were then spread on the surface of a 10 × 10 cm silica gel plate that had been freshly impregnated with an 80 μM solution of substrate **1** or **2** in the same buffer. The silica gel plate was finally placed in a closed Petri dish and the reaction was allowed to proceed at room temperature. In each assay, a fluorescent halo appeared around approximately 4−9 beads (0.1% of approximately 6740 beads) (1−2 beads for (*R*)-**3** and (*S*)-**3**), which were identified as hits (Figure 2E). These active beads as well as a selection of control beads without halo were transferred to analysis vials using a needle and subjected to AAA. The assay including photolysis, reaction, bead picking and AAA was carried out with butyrate ester **1** (4 times) and its analogs **2**, (*S*)-**3** and (*R*)-**3**, allowing for extended incubation times of 6−24 h for these less reactive substrates (2 assays each, only 0.1% hits). From 35 active beads picked in the different assays and analyzed by AAA, 23 returned a readable dendrimer sequences (66%), 10 contained incomplete sequences (29%), and 2 were empty (6%) (Table 1). The analysis of 90 inactive control beads provided 70 dendrimer sequences (78%), 20 incomplete sequences (23%), and no empty beads (Tables S3 and Table S4, Supporting Information).

Analysis of Off-Bead HTS Data. Amino acid occurrences in hits versus nonhits showed interesting trends (Figure 3). For the positions X^4X^3 and X^2X^1, which are designed to form

Table 1. Sequences of Dendrimers on Active Beads As Identified by Off-Bead HTS

assay[a]	no.	dendrimer[b]	X^8	X^7	X^6	X^5	X^4	X^3	X^2	X^1
1	1	**D1**	Ile	Gly	Trp	Pro	His	Leu	Arg	Ser
	2		Tyr	Pro	Glu	Thr	His	Ala	His	Ser
	3		Ile	Gly	Trp	Pro	His	Ser	Arg	Asp
	4		Tyr	Pro	Tyr	Gly	His	Ser	Val	Asp
	5	**D2**	Ile	Pro	Tyr	Phe	His	Ala	His	Asp
	6		Ile	Pro	Tyr	Gly	His	Ala	Val	Leu
	7		Ile	Pro	Trp	Thr	His	Leu	Val	Ser
	8	**D3**	Glu	Thr	Ile	Gly	His	Ala	His	Leu
			Tyr	Pro	Ile	Thr	His	Ala	Arg	Leu
2	10		Ile	Gly	Trp	Pro	His	Ala	Val	Ser
	11	**D4**	Ile	Pro	Trp	Gly	Arg	Ala	His	Asp
	12	**D5**	Trp	Gly	Tyr	Gly	His	Ala	His	Leu
	13	**D6**	Tyr	Phe	Trp	Gly	Val	Leu	His	Ser
	4		Tyr	Thr	Trp	Phe	His	Ser	Arg	Ala
	15		Glu	Pro	Tyr	Thr	His	Leu	Arg	Ala
	6	**D7**	Glu	Gly	Tyr	Gly	His	Ser	Val	Asp
	7	**D8**	Tyr	Pro	Glu	Thr	His	Leu	Arg	Ala
	18	**D9**	Ile	Gly	Glu	Thr	His	Ser	His	Leu
(*S*)-**3**	19	**D10**	Ile	Pro	Trp	Thr	His	Ala	His	Leu
	20	**D11**	Ile	Thr	Tyr	Pro	Cys	Ala	His	Ser
(*R*)-**3**	21	**D12**	Ile	Pro	Trp	Pro	His	Ser	His	Leu
	22	**D13**	Ile	Pro	Trp	Thr	His	Leu	Arg	Leu
	23	**D14**	Ile	Thr	Ile	Pro	His	Asp	Arg	Leu

[a] Assay conditions: the library bead were washed 5 × 3 mL and 2 × 1 h (3 mL) with bis-tris buffer (pH 6.0), dried under vacuum, and then irradiated with 366 nm UV light for 30 min. A silica gel plate was impregnated with a freshly prepared solution of the indicated substrate (80 μM) in bis-tris buffer (pH 6.0), and the photolyzed beads were poured onto the plate surface. The reaction was allowed to proceed at room temperature in a closed Petri dish for 1.5 h (**1**), 6 h (**2**), or 24 h (**3**). Inspection of the beads under a UV lamp (λ = 366 nm) revealed active beads surrounded by a fluorescent halo (Figure 2D), which were picked to be submitted to AAA. [b] The sequences labeled **D1**−**D14** were synthesized, see Table 2.

Table 2. Synthesis of Selected Hits And NonHits from Off-Bead Screening

dendrimer	screen	sequence[a]	yield, mg (%)	MS calcd	MS obsd
D1	**1**	$(AcIG)_8(BWP)_4(BHL)_2BRSNH_2$	17.5 (6%)	4195.8	4196.6
D2	**1**	$(AcIP)_8(BYF)_4(BHA)_2BHDNH_2$	39.5 (13%)	4549.2	4549.0
D3	**1**	$(AcET)_8(BIG)_4(BHA)_2BHLNH_2$	36.3 (13%)	4145.3	4145.6
D4	**2**	$(AcIP)_8(BWG)_4(BRA)_2BHDNH_2$	17.3 (3%)	4318.9	4318.1
D5	**2**	$(AcWG)_8(BYG)_4(BHA)_2BHLNH_2$	3.1 (1%)	4450.7	4449.4
D6	**2**	$(AcYF)_8(BWG)_4(BVL)_2BHSNH_2$	not soluble		
D7	**2**	$(AcEG)_8(BYG)_4(BHS)_2BVDNH_2$	56.2 (12%)	3988.8	3987.6
D8	**2**	$(AcYP)_8(BET)_4(BHL)_2BRANH_2$	49.7 (9%)	4687.0	4686.4
D9	**2**	$(AcIG)_8(BET)_4(BHS)_2BHLNH_2$	21.8 (5%)	3937.2	3936.5
D10	**S-3**	$(AcIP)_8$ $(BWT)_4(BHA)_2BHLNH_2$	30.5 (10%)	4451.1	4453.9
D11	**S-3**	$(AcIT)_8(BYP)$ $_4(BCA)_2BHSNH_2$	44.5 (14%)	4283.9	4283.4
D12	**R-3**	$(AcIP)_8$ $(BWP)_4(BHS)_2BHLNH_2$	38.6 (12%)	4470.2	4469.6
D13	**R-3**	$(AcIP)_8(BWT)$ $_4(BHL)_2BRLNH_2$	21.8 (7%)	4557.4	4556.5
D14	**R-3**	$(AcIT)_8$ $(BIP)_4(BHD)_2BRLNH_2$	25.7 (8%)	4285.0	4284.8
D15	**1**	$(AcIT)_8(BET)_4(BHS)_2BHDNH_2$	4.0 (1%)	4291.6	4291.1
D16	**1**	$(AcET)_8(BEF)_4(BHL)_2BHDNH_2$	53.9 (18%)	4656.7	4656.0
D17	**1**	$(AcEG)_8(BWF)_4(BHS)_2BCDNH_2$	19.1 (7%)	4445.5	4444.8

[a] Amino acids are given in one-letter code. *B* is (S)-2,3-diaminopropionic used as branching point, acylated at the 2- and the 3-amino groups. The N-termini are acetylated (Ac), and the C-terminus at the core is carboxamide.

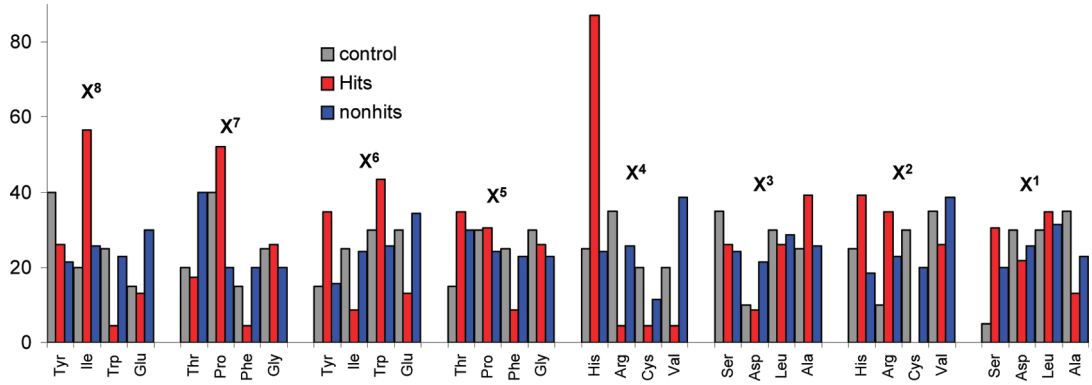

Figure 3. Amino acid occurrence (in percent) at each position in hits, nonhits, and control sequences. The data is calculated from the sequence information in Tables 1, S1, S3, and S4.

the catalytic core, histidine was strongly enriched at position X^4 (87%) and X^2 (39%) and occurred at least once in each hit, as expected from the mechanism of ester hydrolysis.[23] The frequent occurrence of histidine is consistent with the previous on-bead results[10] with substrate **2** in which all 15 hits analyzed had histidine at position X^4 and 11 of

Table 3. Kinetic Parameters of Catalytic Peptide Dendrimers[a]

	butyrate **1**		nonanoate **2**		3-phenylbutyrate (*R*)-**3**		3-phenylbutyrate (*S*)-**3**		dodecanoate **4**	
no.	K_M	k_{cat}/k_{uncat}	K_M	k_{cat}/k_{uncat}	K_M	k_{cat}/k_{uncat}	K_M	k_{cat}/k_{uncat}	K_M	k_{cat}/k_{uncat}
D1	29	250	35[b]	17[b]	180	100	69	30	95[b]	53[b]
D2	210	720	41	93	200	35	680	190	560[b]	93[b]
D4	98	200	93	80	290	56	110	46	57	33
D5	100[b]	33[b]			410[b]	55[b]	140[b]	19[b]		
D10	91	570	12	120	58	38	60	75	88	125
D11	460	150	191	91					360	180
D12	99	600	9	130	34	26	40	56	87	170
D14	680	290	570	260					560	420

[a] Conditions: aq citrate 5 mM buffer pH 5.5, 34°C, 3 h (**1**, **2**, **4**) or 12 h ((*R*)-**3**, (*S*)-**3**), [dendrimer] = 5 μM, [substrate] = 30−1000 μM (8 data points). The reactions were run in 96-well polystyrene plates and followed by fluorescence at λ_{ex} = 450 nm, λ_{em} = 530 nm. Each parameter is the average of three independent determinations, with typical standard deviations of ±10−20%. The background rate, under these conditions, is k_{uncat} (**1**) = $1.57 \cdot 10^{-5}$ min^{-1}, k_{uncat} (**2**) = $2.47 \cdot 10^{-5}$ min^{-1}, k_{uncat} (**3**) = $2.79 \cdot 10^{-5}$ min^{-1}, k_{uncat} (**4**) = $2.00 \cdot 10^{-5}$ min^{-1}. The second-order rate constant for catalysis by 4-methylimidazole is k_2 (**1**) = 0.53 M$^{-1} \cdot$min^{-1}, k_2 (**2**) = 0.54 M$^{-1} \cdot$min^{-1}, k_2 (**3**) = 0.26 M$^{-1} \cdot$min^{-1}, k_2 (**4**) = 0.44 M$^{-1} \cdot$min^{-1}. K_M is in μM. [b] These parameters were determined in bis-tris 10 mM buffer pH 7.0, there was no activity at pH 5.5 in these cases. Dendrimers **D3**, **D7**−**D9**, **D15**−**D17** did not show any activity in these assays. Dendrimer **D13** was insoluble under the assay conditions.

them (73%) also at X^2, which lead to dendrimer **HG3** ((AcIlePro)$_8$(DapIleThr)$_4$(DapHisAla)$_2$DapHisLeuNH$_2$). Analysis of the nonhits in the present assay however showed that histidine also occurred statistically in nonhit sequences at both X^4 (24%) and X^2 (19%), suggesting that its presence was not sufficient for catalysis.

Arginine, which might assist in substrate binding by electrostatic interactions, was almost completely absent at position X^4 and only occurred significantly at position X^2 (35%). This contrasts with the sequences identified previously by an on-bead assay with substrate **1** leading to the identification of dendrimer **RG3** ((AcTyrThr)$_8$-(DapTrpGly)$_4$(DapArgSer)$_2$DapHisSerNH$_2$), in which most hits (50%) contained arginine at position X^4 but often lacked a histidine residue.[10] This false-positive artifact was probably caused by product binding in the absence of true catalysis and was now eliminated by the "off-bead" procedure.

Sequence preferences in hits and nonhits were also apparent for positions X^8X^7 and X^6X^5, which appear in 8 and 4 copies respectively and form the outer, noncatalytic dendritic shell. Isoleucine at position X^8 and proline at position X^7 were found preferentially in hit sequences, with 7 from 23 hits featuring the dipeptide Ile-Pro at position X^8X^7. This residue pair had previously been found as a consensus in the on-bead assay leading to the catalytic sequence **HG3**. On the other hand glutamate was enriched in nonhit sequences (30% Glu at X^8 and 34% Glu at X^6), in particular in histidine containing nonhit sequences. Although glutamate also occurred in hits (13%), their enrichment in nonhits suggested that anionic residues might block catalysis by inhibiting substrate binding through an electrostatic repulsion effect.

Catalytic Properties of Selected Hits and Nonhits. To test if sequences identified by off-bead screening displayed the expected properties, 14 selected hits and 3 nonhits were prepared by SPPS. While many hit sequences were closely related to the previously investigated dendrimers **HG3** and **RG3**, we focused mostly on sequences containing anionic residues to test their role in modulating catalysis. The dendrimers were cleaved from the support and purified by preparative HPLC (Table 2). All but two dendrimers, **D6** and **D13**, which were insoluble, were investigated for catalysis in aqueous buffer with substrates **1**−**4** at pH 5.5

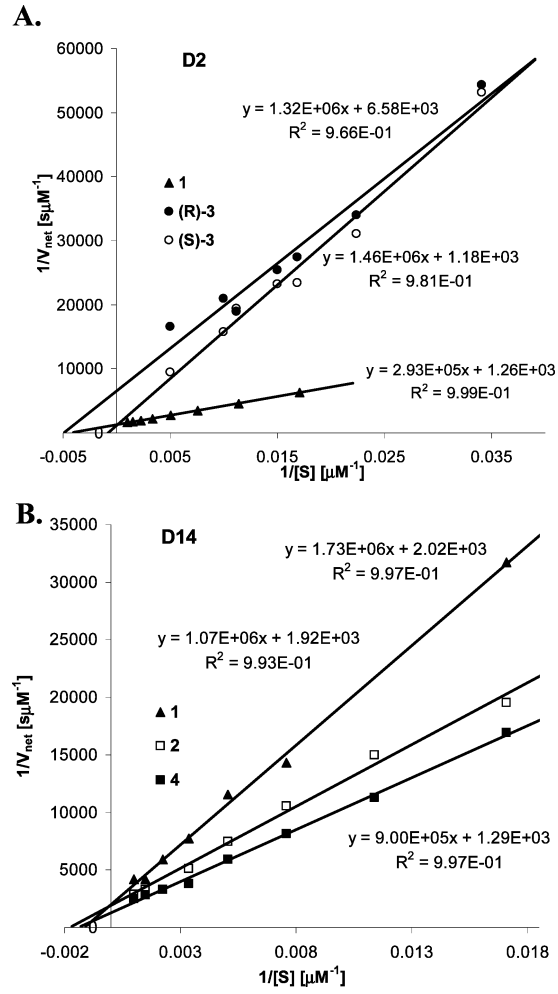

Figure 4. Examples of Michaelis−Menten plots. A. **D2** at pH 5.5; B. **D14** at pH 5.5. Conditions see footnote [a] of Table 3.

and pH 7.0 (Table 3, Table S5, Supporting Information, and Figure 4). Eight of the 12 hits identified were catalytically active, with rate accelerations in the range of k_{cat}/k_{uncat} = 10^1−10^3, and substrate binding in the range of K_M = 10−700 μM, with generally tighter substrate binding (lower K_M values) and higher rate accelerations (k_{cat}/k_{uncat}) at pH 5.5, which is typical for histidine controlling both substrate binding and catalysis as previously reported for **HG3**.

The three nonhits synthesized all had a catalytically competent core $(X^4X^3)_2DapX^2X^1$ with two or three histidine

residues, but displayed eight or twelve glutamate residues at position X^6 and X^8, respectively, which were never found in catalytically active sequences. Similarly, the four inactive hits had four or eight glutamates in the outer branches combined with catalytic histidine residues at the core. The multiple negative charges in the outer branches of the investigated inactive dendrimers probably block catalysis by inhibiting substrate binding by electrostatic repulsion of the substrate's sulfonate groups. Electrostatic interactions controlling substrate binding are consistent with the lower K_M values found in cationic dendrimers with arginine and no anionic side chains, such as dendrimer **D1** (average K_M = 77 μM), and the higher K_M values found in anionic dendrimers, which even loose activity on the less reactive substrates (*S*)-**3** or (*R*)-**3**, such as for dendrimer **D14** (average K_M = 1050 μM).

While the highest catalytic activities were observed with butyrate **1** and nonanoate **2**, the dendrimers also exhibited significant catalytic activity for the more hydrophobic substrates such as the chiral substrates **3** and the dodecanoate **4**, although in some cases only at pH 7.0. Catalysis of dodecanoate **4** hydrolysis is noteworthy because none of our previously reported esterase dendrimers, including **HG3** and **RG3**, showed any activity with this substrate. In the present series, dendrimers **D11**−**D14** identified by screening against (*S*)-**3** and (*R*)-**3** showed the highest activities for dodecanoate **4** among all dendrimers tested. Interestingly, dendrimer **D14** displayed a higher rate acceleration with **4** than with the shorter acyl chain substrates. This dendrimer had a unique sequence combining relatively hydrophobic outer branches with a catalytic core featuring an unusual His-Asp dyad at X^4-X^3 and an Arg-Leu dipeptide at X^2-X1. No significant chiral discrimination was observed in the hydrolysis of the chiral pair 3-phenyl-butyrate ester substrates (*R*)-**3** and (*S*)-**3**, although dendrimer **D4** showed a measurable enantiodifferentiation ($E \approx 1.5$). The absence of significant enantioselectivity probably reflects a high degree of conformational flexibility, which is evident from molecular dynamics simulations of these systems.[10,24]

Discussion

The catalytic parameters of the synthesized dendrimers indicate the overall reliability of the off-bead assay procedure to identify catalytic sequences. However the presence of noncatalytic sequences in the hits, in particular, those with multiple anionic residues, is difficult to explain. The effect could reflect partial side-chain deprotection in the library hiding the inhibitory negative charges during screening, or the presence of catalytic impure sequences in part of the solid supported beads. It should be noted that catalytic sequences such as no. 55 (Table S4, Supporting Information) might appear in the nonhits because of the incomplete photolysis of some of the beads that were outside of the UV-beam during irradiation. Another technical difficulty worth mentioning is that even dispersion of the beads when the dry photolyzed beads are poured over the substrate-impregnated silica gel plate for screening is critical. In our hands, approximately 10−15% of the beads were aggregated in groups of 2−6 beads, precluding hit identification when a fluorescent halo was formed around these beads.

The selection of proper hit sequences from the screening data is a key problem in library HTS. The present study leads to a consensus sequence very similar to our previously reported esterase dendrimer **HG3**. For synthetic work on this project, we rather selected to synthesize strongly anionic hit sequences to clarify the influence of glutamates in catalysis, which lead to several hits being inactive. In retrospect, the statistical analysis of residue occurrences in hits and nonhits (Figure 3) already contained the SAR information leading to **HG3**-like sequences and could have been used to guide hit selection and secure activity in all hits resynthesized. The possibility to analyze and decode multiple hits and nonhits by simple amino acid analysis is therefore an important feature of our off-bead assay.

Our previous report with **HG3** and **RG3** had shown that the outer dendrimer branches do not contribute significantly to catalysis of dendrimers from library **L**, when the residues are mostly hydrophobic (as in **HG3**), but enhance both substrate binding and the catalytic specificity constant when they contain aromatic residues, as in **RG3**.[10] The present SAR study showed that multiple negative charges in these outer dendrimer branches inhibit catalysis when a catalytically competent core is present, and provide an additional example of remote control of catalysis. It should be noted that the presence of an anionic aspartate at the core position X^1 next to the catalytic histidine as in **D2** and **D4** was well tolerated for catalysis, similarly to the fact that switching from a C-terminal carboxylate ester for screening to a C-terminal carboxamide in resynthesized hits did not prevent catalysis to occur.

The off-bead assay selected dendrimers with a high proportion of catalytic histidine residues and tight substrate binding, including sequences bearing anionic residues. On the other hand, our previous on-bead selection led to the frequent selection of arginine residue, and the complete absence of anionic residues.[10] This difference can be interpreted in terms of electrostatic effects on substrate binding controlling the on-bead activity assay by an accumulation/repulsion effect in the bead matrix. Such effects cannot take place in the off-bead assay, where the dendrimer diffuses out of the bead. On the other hand the strong consensus for the hydrophobic dipeptide Ile-Pro at postion X^8-X^7 might reflect a generally higher solubility of the Ile-Pro dendrimers compared to less soluble sequences containing aromatic amino acids, which might not diffuse properly from the beads.

Conclusion

The experiments above illustrate a new and practical off-bead high-throughput in silica screening procedure to test catalysis in OBOC libraries using fluorogenic assays. Although the example chosen concerns a dendrimer combinatorial library tailored toward catalysis of an aqueous ester hydrolysis reaction, the procedure should be transferable to OBOC libraries of other compound types, and to other reaction types that can be assayed by a fluorogenic or colorimetric assay.[25] Aqueous enzyme-like reactions seem

to be generally compatible with the silica gel support. It should also be possible to use the assay with other solvent systems as long as evaporation can be kept under control. Combined with our TAGSFREE library design allowing inexpensive AAA bead decoding,[17a] this simple off-bead catalysis assay should greatly facilitate the exploration of the potential of OBOC libraries for catalysis.

Experimental Section

General. Peptide syntheses were performed manually in syringe reactors. All reagents were either purchased from Aldrich, Fluka (Switzerland), or Acros organics. PYBOP, amino acids and their derivatives were purchased from Advanced ChemTech (USA) or Novabiochem (Switzerland). Amino acids were used as the following derivatives: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-His(Boc)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(*t*Bu)-OH, and Fmoc-Val-OH. FmocDap(Fmoc)-OH was purchased from BACHEM. TG S RAM (loading: 0.23 or 0.24 mmol g^{-1}) and Hydroxymethyl-Photolinker NovaSyn TG (loading: 0.24 mmol g^{-1}) resins were purchased from Novabiochem (Switzerland). Analytical RP-HPLC was performed in a Waters (996 Photo diode array detector) chromatography system using an Atlantis column (dC18, 5 μm, 4.6 × 100 mm, flow rate 1.5 mL min^{-1}). Preparative RP-HPLC was performed in a Waters Prep LC4000 chromatography system using a Delta-Pak C18 column (RP-C18 20 mm, 300 Å pore size, flow rate 80 or 100 mL min^{-1}). Compounds were detected by UV absorption at 214 nm. All RP-HPLC are using HPLC-grade acetonitrile and Milli-Q deionized water. The elution buffers are (A) H_2O with 0.1% TFA, (C) H_2O/MeCN (10:90) with 0.1% TFA, and (D) H_2O/MeCN (40:60) with 0.1% TFA. MS spectra were provided by the Service of Mass Spectrometry of the Department of Chemistry and Biochemistry, University of Bern. Amino acid analyses were provided by the Service of Protein Analysis of the Department of Chemistry and Biochemistry, University of Bern. Kinetic measurements were carried out using a CytoFluor Series 4000 multiwell plate reader from PerSeptive Biosystems. Automated dendrimer syntheses were performed using a PSW 1100 automatic synthesizer from Chemspeed.

(*R*)-8-Phenylbutyryloxypyrene-1,3,6-trisulfonate ((*R*)-3).[26] (*R*)-3-phenylbutyric acid (38 mg, 0.20 mmol) and 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (110 mg, 0.20 mmol) were dissolved in 3 mL DMF. BOP (180 mg, 0.40 mmol) and DIEA (0.1 mL, 0.6 mmol) were added to the mixture and the reaction was stirred during 3 h. The reaction was followed by analytical RP-HPLC. The residue was purified by preparative RP-HPLC (A/D = 90/10 to 50/50 in 30 min, flow = 100 mL min^{-1}). (*R*)-**3** was obtained as a yellow solid (73.3 mg, 61%). ^{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, 39. 44.67, 44, 24.10. MS (ES−): calcd for $C_{26}H_{19}O_{11}S_3$ 603.6; found 603.2.

(*S*)-8-Phenylbutyryloxypyrene-1,3,6-trisulfonate ((*S*)-3). Starting with (*S*)-3-phenylbutyric acid and following the same procedure described for the compound (*R*)-**3**, gave (*S*)-**3** (74.5 mg, 62%) as a yellow solid.

Combinatorial Library by Split-and-Mix. The peptide dendrimer library was prepared from 500 mg resin batch of Hydroxymethyl-Photolinker NovaSyn TG resin (0.24 mmol g^{-1}) divided equally in 4 reactors. The attachment of the first amino acid to hydroxymethyl resin was done by using MSNT/MeIm method.[27] The resin was placed in a syringe, swelled, and covered with dry CH_2Cl_2, and the reactor was flushed with 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 THF. MSNT (5 equiv) and MeIm (3.75 equiv) were added into the flask, and the mixture was added to the syringe. Then the syringe was agitated at room temperature for 1 h. The resin was washed with CH_2Cl_2. The coupling was repeated two times. A sensitive color test (Alizarin-cyanuric chloride test)[28] was used to detect the presence of hydroxyl groups on solid support. The presence of unreacted hydroxyl groups is indicated by red-colored bead. For further couplings, the resin was acylated with one of the four amino acid (3 equiv) in the presence of PyBOP (3 equiv) and DIEA (5 equiv) in NMP. Amino acids and DAP were coupled for 30 min (G0), 1 h (G1), 2 h (G2), and 4 h (G3). After each coupling, the four resin batches were mixed together, vortexed for 1 min, and split into four equally parts. These split-and-mix steps were repeated after each amino acid coupling. After each coupling or deprotection step, the resin was successively washed with NMP, MeOH, and CH_2Cl_2 (3 × 5 mL, with each solvent) and then checked for free amino groups with the TNBS test.[29] Coupling after proline was checked with the chloranil test.[30] If the test indicated the presence of free amino groups, the coupling was repeated. The Fmoc protecting groups were removed with a solution of 20% piperidine in DMF (2 × 10 min), and the solvent was removed by filtration. At the end of the synthesis, the Fmoc protected resin was dried and stored at −18 °C. Just before screening, the Fmoc protecting groups were removed, and the last amino acid was acetylated with acetic anhydride/CH_2Cl_2 (1:1) for 1 h. The side-chain protecting groups were removed with TFA/TIS/H_2O/EDT (93:5:1:1) for 4 h, resulting in a functional dendrimer library on beads. For all the steps, the syringes are covered with aluminum to prevent exposure to light.

Off-Bead Assay. Activity screening was performed by washing the beads (5 × 3 mL) with buffer, swelling the beads (10 mg, 2 × 1 h) in the same aqueous buffered solution (bis-tris 20 mM pH 6.0). After buffer filtration and drying under vacuum, the resin was poured on a Petri dish (diameter: 3 cm), and it was irradiated with an Hg lamp (100 W) for 30 min. A solution of 80 μM of a fluorogenic ester substrate in bis-tris buffer was sprayed on a TLC plate (10 × 10 cm). The beads were poured on the slightly wet TLC, and then it was locked in a Petri dish (diameter: 11 cm) for 1.5 to 24 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 amino acid analysis (AAA).

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

Dendrimer Synthesis. Procedure A. The resin (TG S RAM) 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 or diamino acid (3 equiv) using PyBOP (3 equiv), and DIEA (5 equiv) in NMP. At the end of the synthesis, the resin was acetylated with acetic anhydride/CH_2Cl_2 (1:1) for 1 h. The cleavage was carried out with TFA/TIS/H_2O (94:5:1) or TFA/TIS/H_2O/EDT (93:5:1:1) for Cys sequences for 4 h. The peptide was precipitated with tert-butyl methyl ether then dissolved in a H_2O/MeCN mixture. All dendrimers were purified by preparative RP-HPLC and obtained as TFA salts after lyophilization.

Procedure B. In the automated procedure, the conditions from procedure A were applied. Each acylation was repeated twice. No free-amine test was performed. The unreacted peptides after two repetitions of the coupling were acetylated with acetic anhydride/CH_2Cl_2 (1:1) mixture for 20 min. The dendrimers were cleaved and purified using the described conditions.

Kinetic Measurements. Kinetic measurements were carried out using a CytoFluor Series 4000 multiwell plate reader from PerSeptive Biosystems. Dendrimers (as TFA salts) were used as 15 μM freshly prepared solutions in Milli-Q water. Substrate solutions for the Michaelis−Menten kinetics were prepared by serial dilution by a factor 2/3 (7×) of a freshly prepared 3 mM solution of substrate in Milli-Q water (final concentration on the plate 60−1000 μM). Low K_M determinations were carried using 30−200 μM final substrate concentrations, prepared by serial dilution by factors 2/3 and 1/2. Eight solutions of 8-hydroxypyrene-1,3,6-trisulfonic acid sodium salt ranging from 0 to 100 μM in buffer were used for the calibration curve. Bis-tris 30 mM (pH 7.0) or citrate 15 mM (pH 5.5) was used as buffer, and the pH was adjusted to the desired value with HCl 1 M and NaOH 1 M using a Metrohm 692 pH/ion meter. In a typical experiment, using a multichannel pipet, 40 μL of dendrimer was mixed with 40 μL of buffer, and 40 μL of substrate in a Costar flat-bottom polystyrene 96-well-plate (150 μL). The formation of the product was followed by fluorescence emission using absorbance filter 450/50 and emission filter 530/25. The gain was adjusted using the signal of the calibration curve prior to every experiment (typically a signal 45 000−55 000 for the 100 μM of the product well). The calibration curve (40 μL of product, 40 μL of buffer, and 40 μL of Milli-Q H_2O), and the blank (40 μL of substrate, 40 μL of buffer, and 40 μL of H_2O) were recorded for every experiment in the same time. The temperature inside the instrument was adjusted to 34.0 °C. Kinetic experiments were followed for typically 3 h (to 12 h). The data points were measured every 90 s (or 180 s for 12 h experiment). Fluorescence data were converted to 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, typically between 500 and 2000 s, corresponding to less than 10% conversion.

Determination of Apparent Rate Enhancements and Kinetic Parameters k_{cat} and K_M. V_{cat} is the apparent rate in the presence of dendrimer catalyst; 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 determined from the linear double reciprocal plot $1/V_{net}$ versus 1/[S] (where [S] is the substrate concentration). 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].

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Supporting Information Available. Tables S1−S5, catalytic dendrimers (HPLC traces, MS and kinetics spectra), amino acid analyses of resin beads from off-bead assay, and compound ((*R*)-**3**) characterization. This information is available free of charge via the Internet at http://pubs.acs.org.

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