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Selective Catalysis with Peptide Dendrimers

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Abstract: Peptide dendrimers incorporating 3,5-diaminobenzoic acid **1** as a branching unit (B) were prepared by solid-phase synthesis of $((Ac-A^3)_2B-A^2)_2B-Cys-A^1-NH_2$ followed by disulfide bridge formation. Twenty-one homo- and heterodimeric dendrimers were obtained by permutations of aspartate, histidine, and serine at positions A^1, A^2, and A^3. Two dendrimers catalyzed the hydrolysis of 7-hydroxy-*N*-methyl-quinolinium esters (**2**−**5**), and two other dendrimers catalyzed the hydrolysis of 8-hydroxy-pyrene-1,3,6-trisulfonate esters (**10**−**12**). Enzyme-like kinetics was observed in aqueous buffer pH 6.0 with multiple turnover, substrate binding (K_M = 0.1−0.5 mM), rate acceleration ($k_{cat}/k_{uncat} > 10^3$), and chiral discrimination (E = 2.8 for 2-phenylpropionate ester **5**). The role of individual amino acids in catalysis was investigated by amino acid exchanges, highlighting the key role of histidine as a catalytic residue, and the importance of electrostatic and hydrophobic interactions in modulating substrate binding. These experiments demonstrate for the first time selective catalysis in peptide dendrimers.

Introduction

Enzyme catalysis is based on the productive encounter of substrates with amino acid side-chains and cofactors within catalytic sites.[1] The ultimate understanding of enzyme catalysis should result in the ability to prepare artificial enzymes. This goal has been actively pursued with catalytic antibodies,[2] catalytic polymers,[3] catalytic peptides and protein models,[4−6] and designed metalloproteins and metallopeptides.[7] Recently, we reported a new approach to artificial enzymes by showing that amino acids can be assembled into catalytically functional peptide dendrimers.[8] Dendrimers are ramified structures that adopt a globular or disk-shaped structure as a consequence of topology rather than folding. Dendrimers are being extensively explored in several areas of chemistry and display a range of special properties and functions.[9] Catalysis, for instance, has been obtained in dendrimers by incorporating catalytically active subunits such as metal complexes and cofactors either at the surface or at the core of the dendrimer. The dendrimer is used to provide a particular microenvironment or to increase molecular size and facilitate catalyst separation and recovery.[10] By contrast, our catalytic peptide dendrimer approach aims at studying catalysis and selectivity arising from the interplay between amino acids within the dendrimer structure. While peptide dendrimers have been investigated as protein mimics, antiviral and anticancer agents, vaccines and drug and gene delivery systems,[11] the construction of such catalytic peptide dendrimers had not been studied previously.

Herein, we report a combinatorial series of 21 different peptide dendrimers based on the Fmoc-protected 3,5-diami-

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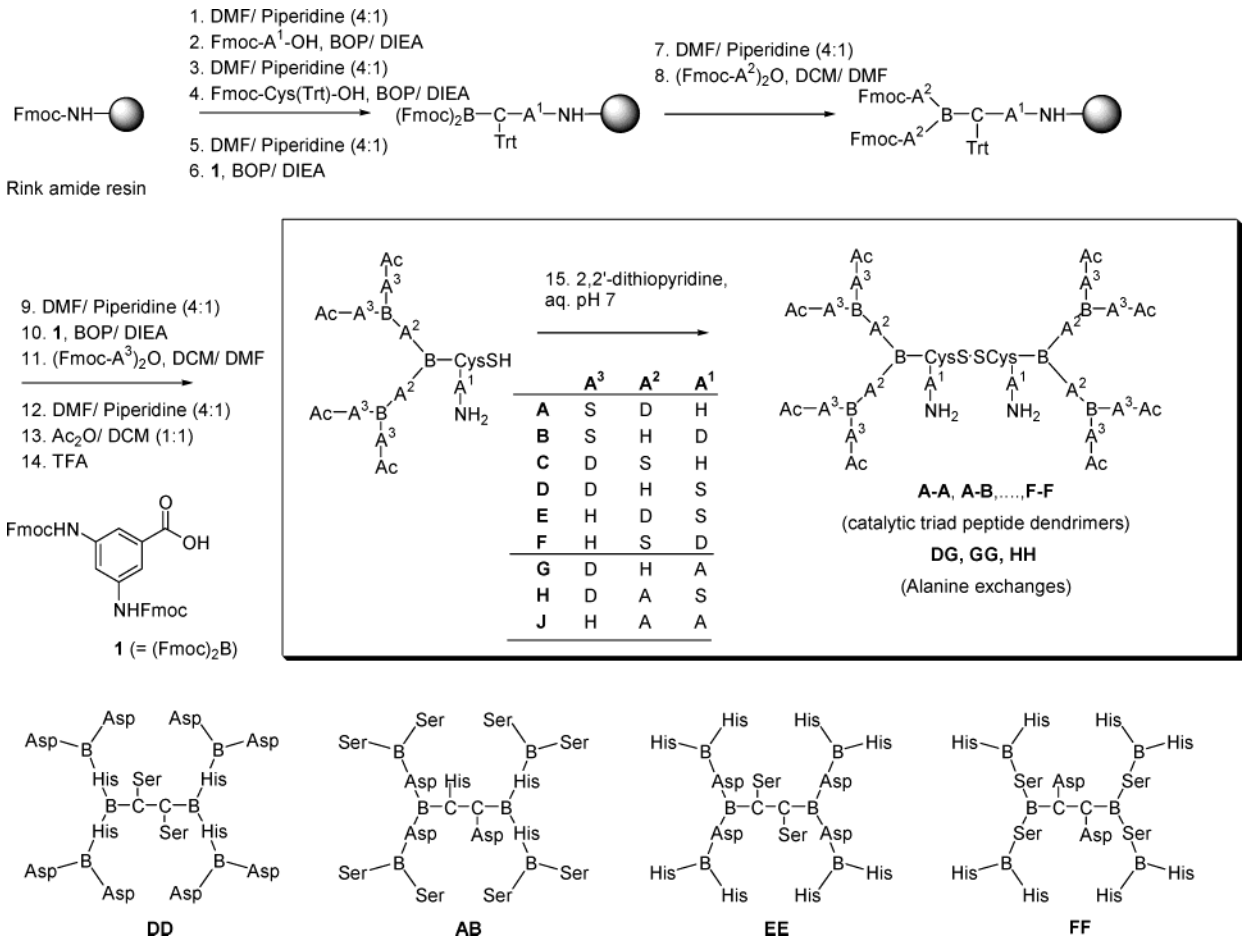
Scheme 1. Solid-Phase Synthesis of Peptide Dendrimers



	A3	A2	A1
A	S	D	H
B	S	H	D
C	D	S	H
D	D	H	S
E	H	D	S
F	H	S	D
G	D	H	A
H	D	A	S
J	H	A	A

nobenzoic acid **1** as a building block for the branching unit and bearing the catalytic triad amino acids serine, histidine, and aspartate at variable positions on the dendrimer branches. Our peptide dendrimers are obtained by dimerization of two second-generation peptide dendrimers by disulfide bond formation (Scheme 1). Two dendrimers were found to catalyze the hydrolysis of 7-hydroxy-*N*-methyl quinolinium esters **2**−**5** in water with significant rate enhancement and chiral discrimination. Two other dendrimers in the series catalyzed the hydrolysis of hydropyrene-trisulfonate esters **10**−**12**. Amino acid exchanges establish the role of individual amino acids in catalysis. These experiments show the first example of chemo- and stereoselective catalysis with peptide dendrimers.

Results and Discussion

The challenge in supramolecular catalysis is to harness productive interactions between functional groups within the supramolecular structure to produce a system with selectivity and catalysis properties not encountered at the level of the building blocks. We chose to investigate the hydrolysis of fluorogenic 7-hydroxy-*N*-methyl quinolinium esters **2**−**8** and 8-hydroxypyrene-1,3,6-trisulfonate esters **10**−**12** for the study of catalytic peptide dendrimers as esterase models (Scheme 2). Both types of esters are well-known substrates for lipases and esterases, and their charged fluorogenic leaving groups offer multiple opportunities to achieve molecular recognition by hydrophobic, π-stacking, or electrostatic interactions. In addition, the hydrolysis of these esters is weakly catalyzed by 4-methyl imidazole in aqueous medium. We reasoned that peptide dendrimers incorporating histidine as one of the building blocks might therefore display catalytic properties for the hydrolysis of these substrates, which might be combined with molecular recognition through interactions with the dendrimer backbone. We would also use serine and aspartate as building blocks to probe the possibility of catalytic-triad-type catalysis.

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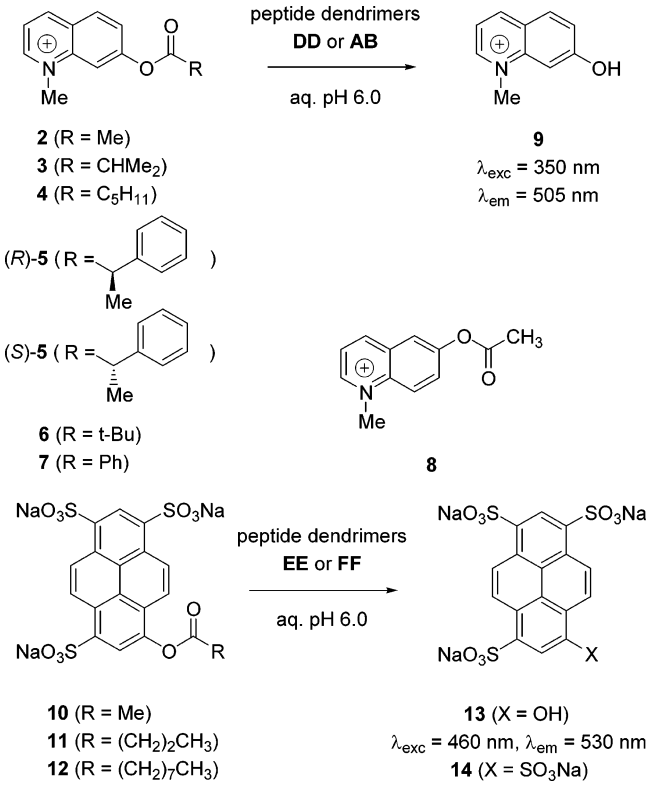
Scheme 2. Peptide Dendrimer-Catalyzed Ester Hydrolysis Reactions



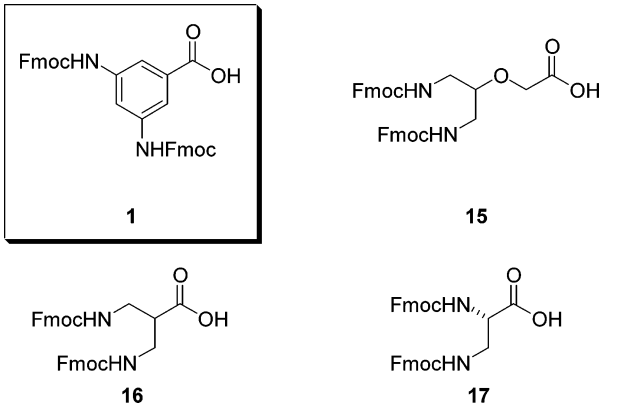


Figure 1. Bis-(Fmoc)-protected diamino acid branching units for peptide dendrimers.

In a previous approach to this problem, we developed the synthesis of peptide dendrimers based on the small aliphatic diamino acids **15**−**17** (Figure 1) as branching units and the dimeric architecture shown in Scheme 1.[8] The dendrimers were functionalized with serine, histidine, and aspartate as variable amino acids at positions A^1, A^2, and A^3. These peptide dendrimers indeed showed catalytic properties; however, catalytic activity was limited to the fluorogenic quinolinium ester substrates **2**−**5** and only occurred when the amino acid histidine occupied the outermost position at the dendrimer surface. Furthermore, no chiral discrimination was observed with chiral substrates. The dendrimers derived from the aliphatic branching units **15**−**17** probably adopted a compact structure barring access of the substrate to the dendrimer core. To circumvent these difficulties, we set out to investigate catalytic peptide dendrimers based on a different branching unit that might result in more open structures allowing substrate access to the dendrimer core. We selected the 3,5-diaminobenzoic acid (as Fmoc-derivative **1**) as the branching unit because it provided a rigid, achiral, and symmetrical structure. Simple modeling considerations indicated that the resulting peptide dendrimers might adopt a relatively open structure, allowing for interaction of inner-shell residues with the substrates.

Synthesis of Peptide Dendrimers. The introduction of 3,5-diaminobenzoic acid as a linker posed a synthetic challenge due to the low reactivity of the aromatic amines for coupling, requiring the development of a new protocol for the solid-phase synthesis. Dendrimer synthesis was carried out on a Rink amide resin by the Fmoc strategy (Scheme 1).[12] The coupling of the first amino acid (A^1) to the resin and the subsequent introduction of cysteine and the Fmoc-protected 3,5-diaminobenzoic acid **1** followed the standard coupling methodology with BOP ((benzotriazol-1-yloxy)-tris-(dimethylamino)-phosphonium-hexafluorophosphate)) as the in situ coupling reagent.[13] However, acylation of the solid-phase bound 3,5-diaminobenzoyl group only gave a mono-acylated product under these conditions, preventing dendrimer growth. The second acylation most likely did not take place due to the inductive deactivation induced by the first acylation. The problem had been circumvented in a previous report of dendrimer synthesis by using a prefunctionalized 3,5-diaminobenzoic acid bis-phenylalanine amide as the dendron.[14] However, this strategy was not practical for the planned synthesis of diverse dendrimer structures. We therefore decided to look for a viable coupling protocol that would avoid the preparation of prefunctionalized units and yield the bis-acylated diaminobenzoic branching as a solid-supported step in the sequence. The use of mixed anhydrides,[15] strong coupling reagents (pyBop,[16] TFFH[17]), BTC/collidine,[18] or $POCl_3$[19] was unsuccessful. Coupling proceeded with excellent yields using the symmetrical anhydrides of *N*-Fmoc amino acids in dichloromethane in the absence of base.[20] The introduction of histidine after the branching unit required the Fmoc−His(Boc)−OH because the symmetrical anhydride of Fmoc−His(trt)−OH (*N*-protected amino acid commonly used in SPPS) did not undergo coupling with the branching unit.

Six monomeric dendrimers incorporating all possible permutations of aspartate, histidine, and serine were prepared by SPPS and obtained in 10−30% yields after purification on preparative HPLC (**A**−**F** in Scheme 1). The monomeric dendrimers were then dimerized using Aldrithiol,[21,22] allowing

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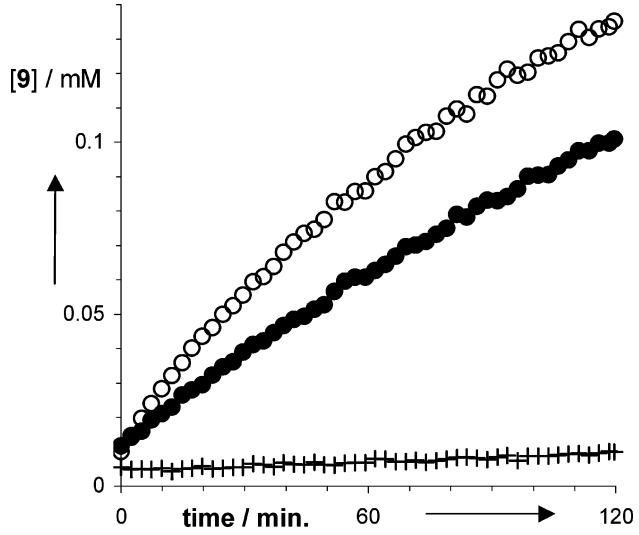

Figure 2. Time-plot of dendrimer-catalyzed hydrolysis of ester **3**. Formation of 7-hydroxy-*N*-methyl quinolinium **9** from isobutyrate **3** with catalytic peptide dendrimer **DD** (●), **GG** (○), and background reaction (+). Conditions: 200 μM substrate **3**, 5 μM dendrimer, 26 °C, in 20 mM aqueous Bis-Tris buffer pH 6.0. The reaction was followed using a SpectraMAX fluorescence plate reader (λ_{exc} = 350 nm, λ_{em} = 505 nm). Fluorescence was converted to product concentration using a calibration curve, which was linear in the concentration range used.

Table 1. Hydrolysis of Quinolinium Ester **2** in the Presence of Peptide Dendrimers[a]

V_{net}/V_{uncat}		A	B	C	D	E	F
A	0.3	0.9					
B	1.4	3.5	0.3				
C	0.3	0.4	1.0	0.7			
D	2.7	0.3	0.2	0.8	7.8		
E	1.3	0.5	1.5	1.5	1.3	1.2	
F	1.2	0.4	1.1	0.6	1.2	1.0	2.2

[a] Conditions: 200 μM **2**, 5 μM dendrimer, 25 °C, 20 mM aqueous Bis-Tris pH 6.0. The ratio V_{net}/V_{uncat} is reported for monomeric dendrimers **A**−**F** (first column) and all combinations of dimers. V_{uncat} is the apparent spontaneous rate of formation of **9** in buffer alone, and $V_{net} = V_{app} - V_{uncat}$, where V_{app} is the apparent rate of formation of **9** in the presence of dendrimer. The reactions were run in 96-well polystyrene half area microtiter plates and followed using a SpectraMAX fluorescence detector with λ_{exc} = 350 nm, λ_{em} = 505 nm. Fluorescence was converted to product concentration using a calibration curve, which was linear in the concentration range used.

the preparation of all 21 possible combinations of disulfide-bridged homo- and heterodimeric dendrimers in yields of 40−60% (**AA**−**FF**). All dendrimers were purified by semipreparative RP-HPLC and characterized by ESI MS.

Dendrimer-Catalyzed Hydrolysis of Quinolinium Esters. The esterolytic activity of the peptide dendrimers derived from diaminobenzoic acid **1** was first investigated using 7-acetoxy-*N*-methyl-quinolinium ester **2** as fluorogenic substrate (200 μM) in aqueous buffer pH 6.0 in the presence of 2.5 mol % catalyst (5 μM dendrimer). Among the six monomers and 21 dimers assayed, dimers **AB** and **DD** showed strong catalytic activity, while all of the other dendrimers showed almost no activity (Figure 2, Table 1). Catalysis was further confirmed by the observation of multiple turnovers. Products were further identified by HPLC analysis of the reaction mixture.

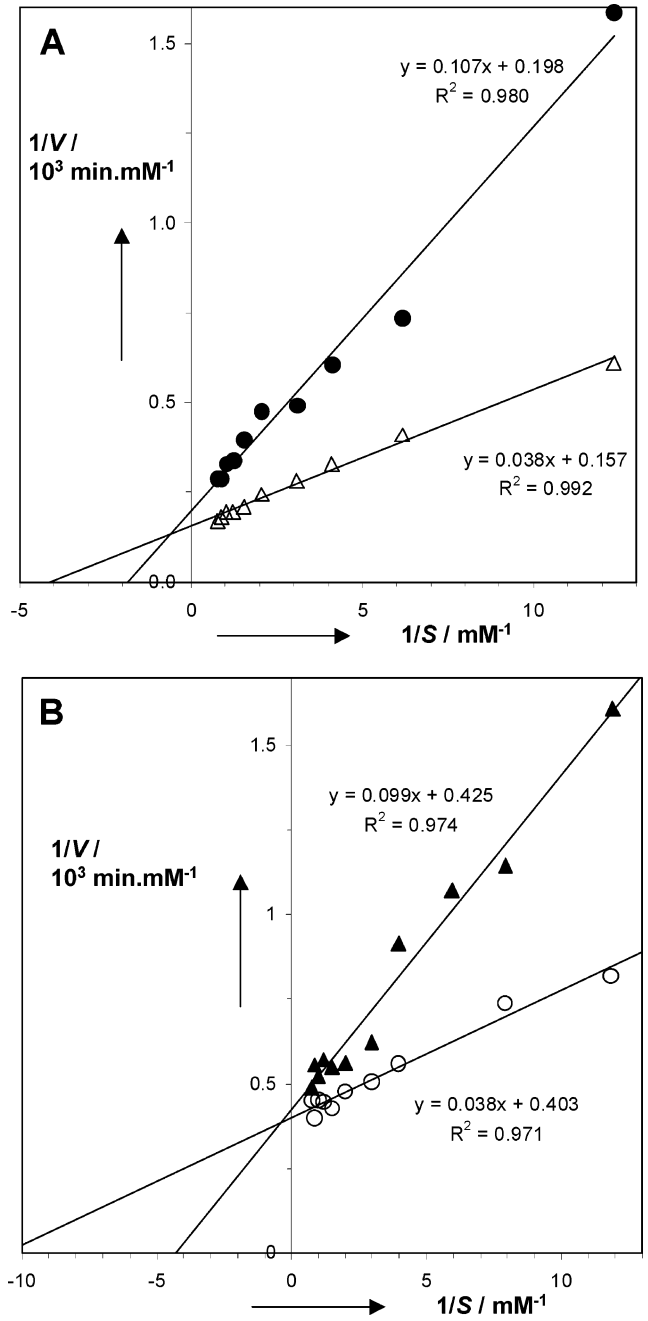

Figure 3. Double reciprocal plot of kinetic data. (A) Hydrolysis of **3** catalyzed by dendrimers **AB** (●) and **DD** (△). (B) Hydrolysis of (*R*)-**5** (▲) and (*S*)-**5** (○) catalyzed by dendrimer **DD**. For conditions, see legend of Table 2.

Peptide dendrimers **AB** and **DD** also catalyzed the hydrolysis of isobutyryl ester **3**, hexanoyl ester **4**, and the chiral 2-phenylpropionate ester **5**. In contrast, there was no reaction with pivalate **6**, benzoate **7**, or the isomeric 6-acetoxy-1-methylquinolinium **8**. The kinetics of hydrolysis followed the Michaelis−Menten model, with Michaelis−Menten constants K_M in the order of 0.1−0.55 mM and k_{cat}/k_{uncat} values in the range of 5×10^2 to 4×10^3 (Table 2, Figure 3). Catalysis was proportional to dendrimer concentration up to 50 μM dendrimer, suggesting that the dendrimers did not aggregate in the buffer at pH 6. Isobutyryl ester **3** was the best substrate for both dendrimers, with a maximum rate enhancement of k_{cat}/k_{uncat} of 4000 for dendrimer **DD**. The most active dendrimer **DD** also

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Table 2. Kinetic Parameters for Dendrimer-Catalyzed Hydrolysis of *N*-Methyl Quinolinium Esters **2**−**5**[a]

		2	3	4	(*S*)-**5**	(*R*)-**5**	E[b]
4-MeIm	k_2 (mM^{-1} min^{-1})	6.7×10^{-3}	4.3×10^{-3}	5.6×10^{-3}	5.0×10^{-3}	4.8×10^{-3}	
	K_{uncat} (min^{-1})	7.7×10^{-4}	1.9×10^{-4}	3.2×10^{-4}	2.9×10^{-4}	2.9×10^{-4}	
DD	K_M (mM)	0.13	0.25	0.14	0.09	0.23	2.8
$(DHS)_2$	k_{cat} (min^{-1})	0.41	0.77	0.45	0.50	0.46	
	k_{cat}/k_{uncat}	540	4000	1400	1740	1600	
	$(k_{cat}/K_M)/k_2$	470	720	570	1100	420	
AB	K_M (mM)	0.17	0.54	0.12	0.10	0.07	1.0
SDH-SHD	k_{cat} (min^{-1})	0.26	0.61	0.27	0.33	0.22	
	k_{cat}/k_{uncat}	340	3200	840	1150	760	
	$(k_{cat}/K_M)/k_2$	230	260	400	660	660	
GG	K_M (mM)	0.17	0.44	0.30	0.08	0.11	1.2
$(DHA)_2$	k_{cat} (min^{-1})	0.23	0.83	0.72	0.21	0.24	
	k_{cat}/k_{uncat}	300	4300	2200	740	830	
	$(k_{cat}/K_M)/k_2$	200	440	430	530	450	

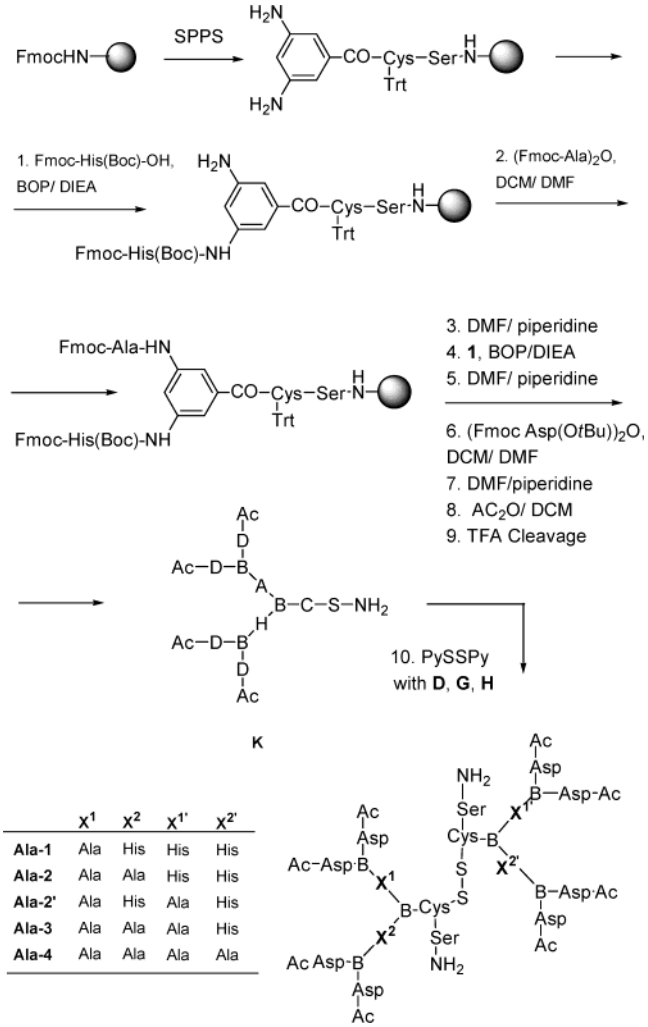
[a] Conditions: 50−800 μM ester substrate, 5 μM dendrimer in 20 mM aqueous Bis-Tris pH 6.0, 25 °C. The kinetic constants given are derived from the linear double reciprocal plots of $1/V_{net}$ versus 1/[S] (Figure 3). [b] $E = (k_{cat}/K_M$ ((*S*)-**5**))/(k_{cat}/K_M ((*R*)-**5**)).

exhibited significant chiral discrimination between both enantiomers of the 2-phenylpropionate ester (*S*)-**5** and (*R*)-**5**, with an enantiomeric ratio $E = 2.8$ in favor of the (*S*)-enantiomer. Interestingly, the catalytic rate constants of both enantiomers were similar, and chiral discrimination was caused mainly by the lower K_M value for (*S*)-**5** ($K_M = 0.090$ mM) as compared to (*R*)-**5** ($K_M = 0.50$ mM).

The catalytic mechanism involved in the dendrimer-catalyzed hydrolysis reaction was investigated next. Because 4-methylimidazole weakly catalyzes the reaction, a conservative mechanistic hypothesis would attribute dendrimer catalysis to the histidine side-chains alone, with the imidazole group acting either as a nucleophilic or as a general-base catalyst and the remainder of the dendrimer structure assisting catalysis by molecular recognition of the substrate. Along these lines, comparison of the specificity constant k_{cat}/K_M for dendrimer catalysis with the second-order rate constant for the reaction with 4-methyl-imidazole k_2 shows that imidazole catalysis is increased by approximately 700-fold within dendrimer **DD**. The rate acceleration is partly due to molecular recognition (K_M), but might also indicate a cooperative effect between two or more histidines, one acting as a nucleophile or general base and another, in protonated form, stabilizing an oxyanion intermediate. This mechanism has been proposed by Baltzer et al. to rationalize a similar enhancement of histidine reactivity in a helical peptide bearing six histidine residues as catalytic side-chains.[5] Alternatively, the large reactivity increase observed could indicate assistance of histidine catalysis by the other two amino acids aspartate and serine, possibly by covalent catalysis in the sense of an enzyme-like catalytic triad.

A series of amino acid exchange experiments were conducted to test these mechanistic hypotheses for the more active homodimeric dendrimer **DD**. An alanine scan study was carried out by sequential alanine replacement of each of the four histidine side-chains at position A^2. Dendrimers with a serine-to-alanine exchange at position A^1 were also prepared. The synthesis of dendrimers incorporating single His−Ala exchanges in position A^2 was realized by exploiting the chemoselectivity observed during synthesis in the acylation of the diaminobenzoic acid branching unit **1** (Scheme 3). Thus, the branching unit following A^1 and cysteine was first coupled under standard conditions with the amino acid histidine, which resulted in a monofunctionalized branch. Coupling with the symmetrical

Scheme 3. Solid-Phase Synthesis of Peptide Dendrimers Incorporating Alanine Residues



anhydride of alanine resulted in coupling of alanine to the second amino-group. Further elaboration then led to the complete monomeric unit bearing one histidine and one alanine at position A^2. It should be noted that this synthetic strategy was free of any stereochemical complexity because no chiral center was created by the monofunctionalization of the diaminobenzoic acid branching unit, this in contrast to the situation which would

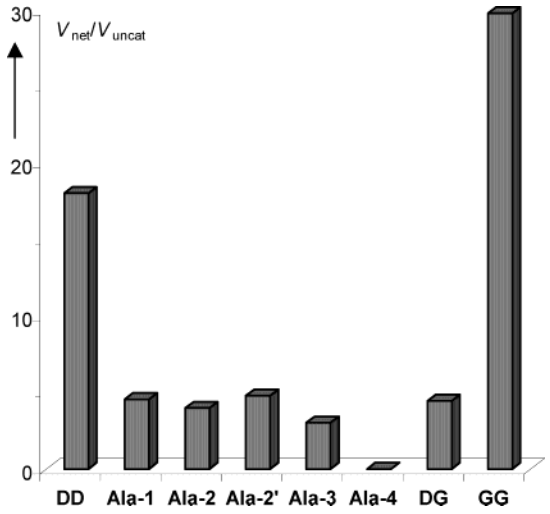


Figure 4. Dendrimer-catalyzed hydrolysis of quinolinium isobutyryl ester **3** in Ala-scan series. The apparent hydrolysis rate of **3** over background (V_{net}/V_{uncat}) is reported for each dendrimer. Conditions: 200 μM **3**, 5 μM dendrimer, 25 °C, 20 mM aqueous Bis-Tris pH 6.0. V_{uncat} is the hydrolysis rate of **3** in buffer alone, and $V_{net} = V_{app} - V_{uncat}$, where V_{app} is the apparent hydrolysis rate of **3** in the presence of dendrimer. See also legend of Table 1.

have arisen by an asymmetric functionalization of the aliphatic branching units **15**−**17**.

Modified dendrimers with one, two, three, and four histidine−alanine replacements were obtained by preparing all possible dimeric combinations involving the single-exchange monomeric dendrimer **K**, monomer **D** (no alanine replacement), and the double histidine−alanine dendrimer **H**. Monomer **G** with Ser−Ala exchange at position A^1 was homodimerized and heterodimerized with monomer **D** to give a dendrimer with one (DHA−DHS) or two (DHA−DHA) Ser−Ala exchanges at position A^1. All resulting dimeric peptide dendrimers were obtained in good yields and purified by semipreparative HPLC as before.

The catalytic efficiency of each Ala-dendrimer was assessed by measuring the apparent hydrolysis rate of the isobutyryl ester **3** at pH 6.0 (Figure 4). Dendrimer **Ala-4** with a 4-fold His−Ala exchange did not show any measurable esterolytic activity, confirming the role of histidine as an essential catalytic residue. Most interestingly, catalytic efficiency was already reduced to 25% by a single His−Ala exchange (**Ala-1**). Moreover, dendrimers with one (**Ala-3**), two (**Ala-2** and **Ala-2′**), or three histidine residues showed a comparable catalytic efficiency. The activity observed in the dendrimer with three histidines exchanged by alanine (**Ala-3**) might be explained by simple nucleophilic catalysis by the single histidine residue. The absence of reactivity increase upon addition of the second and third histidine suggests that these additional histidines cannot operate independently within the dendrimer. The sharp increase in catalytic efficiency upon introduction of the fourth histidine suggests a cooperative effect, for example, in the sense of one histidine residue acting as a nucleophile and the other three histidines providing electrostatic assistance in stabilizing the oxyanion in the tetrahedral intermediate. The rate enhancement effect might also be due to proximity-induced modulation of pK_a values by electrostatic or H-bonding interactions between the different histidines. In any case, the data suggest that catalytic peptide dendrimer **DD** possesses only a single catalytic site that involves a cooperative effect between different amino acid residues.

Table 3. Hydrolysis of Pyrenesulfonate Ester **10** in the Presence of Peptide Dendrimers[a]

V_{net}/V_{uncat}		A	B	C	D	E	F
A	0.3	0.2					
B	0.0	0.2	0.4				
C	−0.1	0.1	0.6	2.0			
D	0.4	0.1	0.1	0.3	2.5		
E	1.9	0.3	1. 4	0.6	0.3	4.5	
F	1.6	0.2	3.6	0.4	0.4	1.4	6

[a] Conditions: 200 μM **10**, 5 μM dendrimer, 25 °C, 20 mM aqueous Bis-Tris pH 6.0. The ratio V_{net}/V_{uncat} is reported for monomeric dendrimers **A**−**F** (first column) and all combinations of dimers. V_{uncat} is the apparent spontaneous rate of hydrolysis of **10** in buffer alone, and $V_{net} = V_{app} - V_{uncat}$, where V_{app} is the apparent rate of hydrolysis of **10** in the presence of dendrimer. The reactions were run in 96-well polystyrene half area microtiter plates and followed using a SpectraMAX fluorescence detector with λ_{exc} = 460 nm, λ_{em} = 530 nm. Fluorescence was converted to product concentration using a calibration curve, which was linear in the concentration range used.

A direct role of the serine residue as a possible nucleophile was ruled out from the data with the Ser−Ala exchange. Indeed, the Ser−Ala dendrimer $(DHA)_2$ (**GG**) showed slightly better catalytic activity than **DD** with quinolinium esters **3** and **4**, whereby both dendrimers showed overall very comparable catalytic efficiencies (Table 2). The absence of a direct role for serine was further supported by the absence of burst-kinetics in the initial phase of the reaction. In addition, the catalytic dendrimers **DD** and **GG** appeared unchanged upon HPLC-analysis of the reaction mixture, ruling out the existence of a long-lived covalent intermediate. The much lower activity of **DG** with only one Ser−Ala replacement was surprising and suggested that additional effects were at play in catalysis.

Given the nonessential role of serine at position A^1, the absence of any catalytic effect in dendrimer **BB** bearing four catalytic residues at position A^2 like dendrimer **DD**, but having aspartate at A^1 and serine at A^3, suggests that the aspartate residues at position A^3 also play a decisive role. These anionic residues might be necessary for molecular recognition of the cationic quinolinium ester substrates **2**−**5** by dendrimer **DD**. Such an effect, however, does not explain the activity of peptide dendrimer **AB**, which has eight serine residues at the outer position A^3.

Dendrimer-Catalyzed Hydrolysis of Hydroxypyrene-trisulfonate Esters. Screening of the peptide dendrimers series using the anionic fluorogenic substrate 8-acetoxypyrene-1,3,6-trisulfonate **10** identified the two homodimers **EE** and **FF** as being catalytically active for this substrate (Table 3). Both dendrimers displayed histidine as the outer position A^3. An even stronger activity was observed with the corresponding butyrate ester **11**. Catalysis followed the Michaelis−Menten model with K_M in the order of 0.1 mM and k_{cat}/k_{uncat} values from 400 to 900 (Table 4). The corresponding nonanoyl ester **12** was also hydrolyzed by the dendrimers; however, the K_M value was very small ($K_M \ll 10$ μM) and could not be determined precisely. The lower K_M values with longer acyl chain in the series **10**-**11**-**12** suggest that substrate binding involves hydrophobic interactions between the dendrimer and the aliphatic chain of the acyl group.

The catalytic dendrimers **EE** and **FF** were between 210-fold and 670-fold more reactive toward the pyrene-trisulfonate

Table 4. Michaelis−Menten Parameters for the Most Active Dendrimers on Pyrene Substrates **10**−**12**[a]

		10	11	12[b]
4-MeIm	k_{uncat} (min^{-1})	8.5×10^{-5}	4.4×10^{-5}	4.9×10^{-5}
	k_2 (mM^{-1} min^{-1})	1.1×10^{-3}	8.1×10^{-4}	8.2×10^{-4}
EE	K_M (mM)	0.20	0.23	<0.01
$(HDS)_2$	k_{cat} (min^{-1})	0.036	0.039	0.011
	k_{cat}/k_{uncat}	410	880	
	$k_{cat}/K_M/k_2$	170	210	
FF	K_M (mM)	0.10	0.07	<0.01
$(HSD)_2$	k_{cat} (min^{-1})	0.055	0.038	0.0098
	k_{cat}/k_{uncat}	620	853	
	$k_{cat}/K_M/k_2$	520	670	
JJ	K_M (mM)	0.07	0.03	<0.01
$(HAA)_2$	k_{cat} (min^{-1})	0.08	0.037	0.0096
	K_{cat}/k_{uncat}	900	840	
	$k_{cat}/K_M/k_2$	1100	1500	

[a] Conditions: 50−800 μM ester substrate **10**−**12**, 5 μM dendrimer in 20 mM aqueous Bis-Tris pH 6.0, 25 °C. The kinetic constants given are derived from the linear double reciprocal plots of $1/V_{net}$ versus 1/[S]. See also legend of Table 3. [b] Only V_{max} was observed with this substrate.

substrate **11** as compared to free 4-methyl imidazole, as judged by the ratio of the specificity constant for dendrimer catalysis k_{cat}/K_M to the second-order rate constant of 4-imidazole catalysis k_2. The presence of histidine residues at the surface position A^3 of both catalytic dendrimers suggested that catalysis was largely due to the imidazole side-chains. The protonated histidine side-chains probably assist substrate binding by electrostatic binding to the sulfonate groups, while the free base form acts as a general base or nucleophilic catalyst for hydrolysis. Hydrolysis of these pyrene-sulfonate esters is also catalyzed by self-assembled pores similarly bearing multiple histidine side-chains.[23]

The role of histidine residues in catalysis was confirmed by exchanging all other residues to alanine to give a similarly catalytically active dendrimer. In fact, the $(HAA)_2$ dendrimer **JJ** catalyzed the reaction even more efficiently than the parent dendrimers **EE** or **FF** in terms of both rate acceleration k_{cat}/k_{uncat} and specificity constant k_{cat}/K_M (Table 4). Interestingly, substrate binding as measured by K_M decreased in the series **EE-FF-JJ**. The trend is consistent with significant interactions between the substrates and the dendrimer core (residues A^1 and A^2) in terms of either electrostatic substrate repulsion (lower K_M with decreasing number of negatively charged aspartates) or hydrobic substrate binding (lower K_M with increasing number of hydrophobic residues).

The electrostatic component in binding was supported by the observation of competitive inhibition of catalysis by the quadruply negatively charged 1,3,6,8-pyrene-tetrasulfonic acid **14** (Scheme 2). This compound inhibited dendrimer catalysis with $K_I = 130$ μM for **EE** and $K_I = 30$ μM for **FF**. These inhibition constants are too large to be compatible with a transition state analogue-type inhibition. Indeed, the calculated transition state dissociation constants of dendrimers **EE**, **FF**, and **JJ** for the pyrene-trisulfonate hydrolysis are in the range of $K_{TS} = K_M/(k_{cat}/k_{uncat}) = 0.08-0.25$ μM. Furthermore, 1,3,6,8-pyrene-tetrasulfonic acid **14** did not show any inhibition for the dendrimer **JJ**, suggesting that hydrophobicity-driven substrate binding was more important than electrostatic interactions in this case.

Comparison with Other Esterase Models. The catalytic peptide dendrimers above can be compared in efficiency and selectivity to other esterase models recently reported in the literature. A four-helix bundle esterase catalyst with six histidine residues described by Baltzer et al. was reported to hydrolyze mono-*p*-nitrophenyl fumarate ($K_M = 1$ mM), a substrate similar in reactivity to the ester substrates above, with a catalytic proficiency of $k_{cat}/K_M/k_2 = 230$ relative to 4-methyl imidazole at pH 5.1.[5b] This four-helix bundle catalyst exhibited a chiral discrimination of $E = 2$ for the corresponding D- and L-norleucine nitrophenyl ester, which is similar to the selectivity observed with dendrimer **DD** and the chiral esters (*R*)-**5** and (*S*)-**5**. Another four-helix bundle selected for folding from a library by Hecht et al. and bearing 12 histidine and 8 surface lysine residues was recently reported to catalyze the hydrolysis of nitrophenyl acetate with a catalytic proficiency of $k_{cat}/K_M/k_2 = 100$ over 4-methyl imidazole at pH 7.0 and $K_M = 3$ mM, corresponding to a rate acceleration of $k_{cat}/k_{uncat} = 8700$ above background at that pH.[6b] The high K_M values of the nitrophenyl ester substrates observed with these four-helix bundles were attributed to the fact that substrate binding was not designed specifically.[6b] As discussed above, substrate binding by our catalytic peptide dendrimers (0.03 mM < K_M < 0.54 mM) is reinforced by electrostatic complementarity between the charged leaving group and the amino acid side-chains. An esterase active site computationally engineered to bind and catalyze the hydrolysis of nitrophenyl acetate at the surface of thioredoxin with a single histidine residue exhibited catalysis at pH 7.0 ($k_{cat}/K_M/k_2 = 25$, $k_{cat}/k_{uncat} = 180$) with a tighter substrate binding ($K_M = 0.17$ mM).[6a] Catalysis and chiral discrimination with relatively tight and specific substrate binding have been amply demonstrated by various catalytic antibodies raised against transition state analogues.[2] These antibodies operate with rate accelerations in the range $k_{cat}/k_{uncat} = 10^2-10^4$ and 0.006 mM < K_M < 0.2 mM. In contrast to artificial systems exhibiting nucleophilic histidine catalysis, catalytic antibodies may also display other reaction mechanisms involving direct water attack or formation of an acyl-antibody intermediate at a catalytic serine side-chain.

Experimental Section

Materials and Methods. All reagents were either purchased from Aldrich or Fluka Chemica (Switzerland) or synthesized following literature procedures. Amino acids and their derivatives were purchased from Senn Chemicals or Novabiochem (Switzerland). BOP and DIC

(23) Baumeister, B.; Sakai, N.; Matile, S. *Org. Lett.* **2001**, *3*, 4229−4232.

were from Fluka. Rink amide resin was purchased from Novabiochem (Switzerland). All solvents used were analytical grade. Peptide syntheses were performed manually in a glass reactor. Analytical RP-HPLC was performed in a Waters (996 Photodiode array detector) chromatography system using a chromolith performance RP-18e, 4.6 × 100 mm, flow rate 3 mL min^{-1} column. Compounds were detected by UV absorption at 214 and 234 nm. 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 system from Millipore (flow rate 100 mL min^{-1}, gradient 1% min^{-1} CH_3CN). Semipreparative RP-HPLC (flow rate, 4 mL min^{-1}; eluant A, water and 0.1% TFA; eluant B, acetonitrile, water, and TFA (3/2/0.1%)); column, Vydac 218 TP (1.0 cm×25 cm, 300 Å pore size). Compounds were detected by UV absorption at 220 nm. Chromatography (flash) was performed with Merck silica gel 60 (0.040 ± 0.063 mm). TLC was performed with fluorescent F254 glass plates. Fluorescence measurements were carried out with a spectraMAX fluorescence detector. NMR spectra were recorded on a Bruker-AC-300 (^1H, 300 MHz; ^{13}C, 75 MHz); MS was provided by Dr. Thomas Schneeberger (University of Bern, Switzerland).

3,5-Bis-(9*H*-fluoren-9-yloxycarbonylamino)benzoic Acid (1). 3,5-Diaminobenzoic acid (0.5 g, 3.28 mmol) was dissolved in a 10% solution of $NaHCO_3$ (25 mL, 23.6 mmol). Dioxane (7.5 mL) was added, and the mixture was stirred in an ice−water bath. 9-Fluoromethyl chlorocarbonate (1.87 g, 7.23 mmol) dissolved in dioxane (25 mL) was added dropwise, and stirring was continued at 0 °C for 1 h and then at room temperature for 8 h. The reaction mixture was then poured into water (100 mL) and extracted (2×) with diethyl ether. The aqueous solution was cooled in an ice−water bath and acidified with 3 N HCl solution until pH 2. The aqueous phase was extracted (3×) with ethyl acetate. The organic layers were combined, washed (1×) with a saturated solution of NaCl, dried under sodium sulfate, and concentrated under vacuum. The product was obtained as a white powder by precipitation of the crude material in a mixture of diethyl ether/hexane with a yield of 72%. mp: 244−246 °C. ^1H NMR (300 MHz, DMSO-d_6) δ (ppm): 4.33 (t, J = 7.0 Hz, 2H), 4.47 (d, J = 7.0 Hz, 4H), 7.3−7.5 (m, 8H), 7.73−7.75 (m, 6H), 7.92 (d, 4H), 8.05 (s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 51.76, 70.90, 118.60, 125.40, 130.42, 132.34, 132.93, 144.94, 145.98, 148.93, 158.56. MS (+TOF): 597.2157.

General Procedure. Coupling of the First Amino Acids on Rink Amide Resin. The Rink amide resin (0.61 mmol/g) was acylated with 3 equiv of *N*-Fmoc amino acid or bis-Fmoc diaminobenzoic acid in the presence of 3 equiv of BOP (benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate and 5 equiv of DIEA (*N*,*N*′-diisopropylethylamine). After 45 min, the resin was washed (2× each) with DMF, MeOH, and DMC (dichloromethane) and then controlled with the TNBS (trinitrobenzenesulfonic acid) test.

Fmoc Removal. The Fmoc protecting group was removed with a solution of piperidine in DMF (1:4) for 3 min. After filtration, the procedure was repeated for 7 min and then washed (2× each) with DMF, MeOH, and DCM.

Incorporation of the *N*-Fmoc Amino Acid on the Free Amines of the Branching Unit. To a solution of *N*-Fmoc amino acid (12 equiv relative to resin loading for the first generation and 24 equiv for the second one) in dry DCM (a few drops of DMF may be required to aid complete dissolution) under nitrogen atmosphere was added diisopropylcarbodiimide (6 or 12 equiv relative to resin loading). The reaction mixture was stirred for 20 min at 0 °C. At the same time, the resin was suspended in dry DCM. After filtration of the resin, the symmetrical anhydride solution was added with a few drops of DMF to the resin, and the resin was shaken 4 h for the first generation and overnight for the second one. The resin was then filtered and washed as before. The coupling efficiency was checked with the chloranil test.

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. The resin was then dried under vacuum and stored at −4 °C.

TFA Cleavage. The cleavage was carried out using a TFA (trifluoroacetic acid)/EDT (1,2-ethanedithiol)/H_2O/TIS (triisopropylsilane) 94.5/2.5/2.5/1 solution for 4 h. The peptide was precipitated with methyl *tert*-butyl ether and then dissolved in a water/acetonitrile mixture. All of the dendrimers were purified by preparative HPLC.

General Procedure of Homodimerization. The monomeric dendrimer X (1 mg) was dissolved in water (25 μL), and a methanolic solution of Aldrithiol was added (45.4 mM, 0.45 equiv). The pH of the solution was adjusted to 8 with a $(NH_4)HCO_3$ buffer solution (20 mM). The solution was stirred for 30 min and then acidified with one drop of TFA. The homodimer XX was purified by semipreparative RP-HPLC.

General Procedure of Heterodimerization. To a solution of dendrimer X (1 mg/25 μL) in water was added a methanolic solution of Aldrithiol (45.4 mM, 5 equiv). The thiol-activation was followed by HPLC, and, after the evaporation of methanol, the Aldrithiol excess was removed by extraction (3×) with DCM. To this activated dendrimer solution was added a solution of dendrimer Y (1 mg/25 μL, 1 equiv) in water, and the pH of the solution was adjusted to 8 with a $(NH_4)HCO_3$ buffer solution (20 mM). The solution was stirred during 30 min and then acidified with one drop of TFA. The heterodimer XY was purified by semipreparative RP-HPLC.

Yields and characterizations of monomers and the corresponding active dimer dendrimers are described below. Yields, purification methods, and characterizations of all dendrimers are found in the Supporting Information. When not indicated, conditions for the analytical RP-HPLC are 100% A to 50% A in 10 min.

Dendrimer A. Starting with 100 mg of Rink amide resin (0.61 mmol/g), the sequence $((Ac-Ser)_2-B-Asp)_2-B-Cys-His-NH_2$ was obtained as a colorless foamy solid after preparative HPLC purification (8.7 mg, 10%). RP-HPLC: t_R = 5.95 MS (ES+): calcd for $C_{58}H_{71}N_{17}O_{23}S$, 1405.75; found, 1405.46.

Dendrimer B. Starting with 100 mg of Rink amide resin (0.61 mmol/g), the sequence $((Ac-Ser)_2-B-His)_2-B-Cys-Asp-NH_2$ was obtained as a colorless foamy solid after preparative HPLC purification (10.7 mg, 12%). RP-HPLC: t_R = 6.09 MS (ES+): calcd for $C_{60}H_{72}N_{18}O_{22}S$, 1427.88; found, 1428.48.

Dendrimer C. Starting with 100 mg of Rink amide resin (0.61 mmol/g), the sequence $((Ac-Asp)_2-B-Ser)_2-B-Cys-His-NH_2$ was obtained as a colorless foamy solid after preparative HPLC purification (17.0 mg, 19%). RP-HPLC: t_R = 5.84 MS (ES+): calcd for $C_{60}H_{71}N_{17}O_{25}S$, 1461.45; found, 1461.63.

Dendrimer D. Starting with 150 mg of Rink amide resin (0.61 mmol/g), the sequence $((Ac-Asp)_2-B-His)_2-B-Cys-Ser-NH_2$ was obtained as a colorless foamy solid after preparative HPLC purification (38.2 mg, 29%). RP-HPLC: t_R = 6.17 MS (ES+): calcd for $C_{63}H_{73}N_{19}O_{24}S$, 1511.48; found, 1512.00.

Dendrimer E. Starting with 100 mg of Rink amide resin (0.61 mmol/g), the sequence $((Ac-His)_2-B-Asp)_2-B-Cys-Ser-NH_2$ was obtained as a colorless foamy solid after preparative HPLC purification (10.0 mg, 11%). RP-HPLC: t_R = 5.87 MS (ES+): calcd for $C_{60}H_{71}N_{17}O_{25}S$, 1555.54; found, 1555.88.

Dendrimer F. Starting with 200 mg of Rink amide resin (0.61 mmol/g), the sequence $((Ac-His)_2-B-Ser)_2-B-Cys-Asp-NH_2$ was obtained as a colorless foamy solid after preparative HPLC purification (30.0 mg, 16%). RP-HPLC: t_R = 5.76 MS (ES+): calcd for $C_{66}H_{77}N_{23}O_{19}S$, 1527.55; found, 1528.13.

Dendrimer G. Starting with 150 mg of Rink amide resin (0.61 mmol/g), the sequence $((Ac-Asp)_2-B-His)_2-B-Cys-Ala-NH_2$ was obtained as a colorless foamy solid after preparative HPLC purification (19 mg, 14%). RP-HPLC: t_R = 6.23 MS (ES+): calcd for $C_{63}H_{73}N_{19}O_{23}S$, 1495.48; found, 1495.63.

Dendrimer H. Starting with 100 mg of Rink amide resin (0.61 mmol/g), the sequence $((Ac-Asp)_2-B-Ala)_2-B-Cys-Ser-NH_2$ was obtained as a colorless foamy solid after preparative HPLC purification

(8.5 mg, 10%). RP-HPLC: t_R = 7.36 MS (ES+): calcd for $C_{57}H_{69}N_{15}O_{24}S$, 1379.44; found, 1381.38.

Dendrimer K. Starting with 150 mg of Rink amide resin (0.61 mmol/g), the sequence ((Ac−Asp)$_2$−B−His)−((Ac−Asp)$_2$−B−Ala)−B−Cys−Ala−NH_2 was obtained as a colorless foamy solid after preparative HPLC purification (12.6 mg, 9.5%). RP-HPLC: t_R = 6.68 MS (ES+): calcd for $C_{60}H_{71}N_{17}O_{24}S$, 1445.46; found, 1445.63.

Dendrimer J. Starting with 50 mg of Rink amide resin (0.61 mmol/g), the sequence ((Ac−His)$_2$−B−Ala)$_2$−B−Cys−Ala−NH_2 was obtained as a colorless foamy solid after preparative HPLC purification (5 mg, 11.3%). RP-HPLC: t_R = 6.61 MS (ES+): calcd for $C_{65}H_{77}N_{23}O_{15}S$, 1451.57; found, 1452.25.

Dendrimer AB. 0.3 mg, 15%, RP-HPLC: t_R = 6.12 MS (ES+): calcd for $C_{119}H_{146}N_{36}O_{44}S_2$, 2832.83; found, 2831.88.

Dendrimer DD. 0.6 mg, 60%, RP-HPLC: t_R = 5.76 MS (ES+): calcd for $C_{136}H_{142}N_{38}O_{48}S_2$, 3020.96; found, 3022.25.

Dendrimer EE. 0.6 mg, 60%, RP-HPLC: t_R = 6.13 MS (ES+): calcd for $C_{134}H_{150}N_{46}O_{40}S_2$, 3109.08; found, 3109.88.

Dendrimer FF. 0.4 mg, 40%, RP-HPLC: t_R = 6.16 MS (ES+): calcd for $C_{134}H_{150}N_{46}O_{40}S_2$, 3053.10; found, 3054.25.

Dendrimer GG. 0.5 mg, 50%, RP-HPLC: t_R = 6.24 MS (ES+): calcd for $C_{126}H_{144}N_{38}O_{46}S_2$, 2988.96; found, 2989.88.

Dendrimer JJ. 0.5 mg, 50%, RP-HPLC: t_R = 7.17 MS (ES+): calcd for $C_{130}H_{152}N_{46}O_{30}S_2$, 2901.14; found, 2902.50.

Kinetic Measurements. The kinetic measurements were carried out by using a SPECTRAMax fluorescence detector with preset values of the excitation and emission wavelengths corresponding to the measured substrate (pyrene substrates λ_{ex} = 460 nm, λ_{em} = 530 nm, *N*-methyl quinolinium substrates λ_{ex} = 350 nm, λ_{em} = 505 nm) at 25 °C. Assays were followed in individual wells of round-bottom polystyrene 96-well plates (Costar). Kinetic experiments were followed for 2 h. The dendrimers were kept at −20 °C in 1 mM stock solution in B (acetonitrile/water: 1/1). Dendrimer stock solutions were freshly diluted to 0.05 mM solution in 20 mM aqueous Bis-Tris pH 6.0. The Bis-Tris buffer pH 6.0 was prepared using MilliQ deionized water, the pH being adjusted with aqueous NaOH and aqueous HCl solutions.

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 aqueous Bis-Tris pH 6.0 (20 mM) was first added in a well, and then 2.5 μL of a dendrimer solution (0.05 mM in aqueous Bis-Tris pH 6.0, final concentration in the well: 5 μM), and last 2.5 μL of substrate solution (2 mM in B, final concentration in the well: 200 μM). The rate observed under these conditions is the apparent rate V_{app}. V_{uncat} is the rate observed with 22.5 μL of aqueous Bis-Tris pH 6.0 (20 mM) and 2.5 μL of substrate solution (2 mM in B, 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 50, 75, 100, 150, 200, 300, 400, 500, 600, 700, and 800 μM substrate, 20 mM Bis-Tris, 25 °C. Kinetic parameters such as maximum velocity (V_{max}) and the Michaelis constant (K_M) were determined by the least-squares method from a Lineweaver−Burk plot. The reaction rate with 4-methylimidazole (4-MeIm) was obtained under the same conditions with 50, 75, 100, 150, 200, 300, 400, 500, 600, 700, and 800 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. The inhibition constant K_I was obtained from the Dixon-plot $1/V_{net}$ versus [I] measured similarly with 5 μM dendrimer, 200 μM substrate, and 0, 10, 20, 40, 60, 80, 100, 200, 400, 600, 800, and 1000 μM inhibitor, 20 mM Bis-Tris, 25 °C. Experiments were repeated twice and gave reproducible values with ±10% error.

Conclusion

Two pairs of catalytic peptide dendrimers based on catalytic triad amino acids and the diaminobenzoic acid branching unit **1** have been identified. Dendrimers **AB** and **DD** catalyze the hydrolysis of 7-hydroxyquinolinium esters **2**−**5**, while dendrimers **EE** and **FF** catalyze the hydrolysis of pyrene-trisulfonate esters **10**−**12**. The orthogonal reactivities of these dendrimers are noteworthy and highlight the selectivity of catalysis. This selectivity is made possible by molecular recognition of the substrates, which is probably mediated by electrostatic interactions between surface aspartates and the cationic quinolinium group in the case of dendrimer **DD**, and between surface histidines and the anionic trisulfonate group with dendrimers **EE** and **FF**. In the latter case, a hydrophobic component of substrate binding is also evident in the lower K_M values observed for substrates with longer acyl chains. On the other hand, the observation of significant chiral discrimination in the hydrolysis of chiral substrate **5** by dendrimer **DD** highlights the existence of precise molecular interactions between substrate and dendrimers. The observation of catalysis with the heterodimeric dendrimers **AB** also suggests that additional interactions leading to catalysis are possible within the dendrimer framework. For dendrimer **DD**, the loss of 75% of activity upon exchange of a single histidine residue (**Ala-1**) clearly shows that catalysis involves cooperativity between different amino acids within the dendrimer.

Our goal was to produce a dendritic catalyst that would be active in aqueous medium, the most relevant medium for the study of enzyme mimics. This stands in contrast to many supramolecular systems based on hydrogen-bonding interactions for both structure and molecular recognition, which are generally investigated in organic solvent.[24,25] Our design was based on topology by providing a dendrimeric framework enforcing spatial proximity between amino acid side-chains irrespective of the actual nature of each amino acid and therefore allowing for the preparation of a series of peptide dendrimers. The peptide dendrimer framework was found to be very robust to amino acid substitution and well-suited for aqueous catalysis. Indeed, the peptide dendrimers were all water soluble and did not form aggregates, as evidenced by the proportionality of catalysis to dendrimer concentration. This is also supported by the absence of any signals in light scattering measurements (data not shown). Dendrimer synthesis combined divergent solid-phase synthesis of the monomeric dendrimers followed by convergent disulfide-bridge formation to the dimers and provided a versatile and rapid access to dendrimers in excellent yields and purity. This factor not only enabled the assembly of a dendrimer family from which active members could be identified, but also the preparation of modified dendrimers as a means to understand the role of individual amino acids in catalysis.

The use of 3,5-diaminobenzoic acid **1** as a branching unit was thought to provide dendrimers with open structures, allowing substrate access to the inner residues at positions A^1 and A^2. This hypothesis seemed to be confirmed by the observation of catalysis and chiral discrimination with dendrimer **DD** bearing catalytic histidines at position A^2. In any case, this is the first example of a catalytic peptide dendrimer capable of

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selectivity in terms of both substrate type and chiral discrimination. The rate enhancements obtained are up to 4000-fold, which compares well with catalytic peptides and most catalytic antibodies reported for similar esterolytic reactions. The straightforward synthetic access can be used to prepare a large variety of such dendrimers by variation of the amino acids at positions A^1, A^2, and A^3, which corresponds to millions of different dendrimers counting only proteinogenic amino acids. A systematic investigation of these dendrimers for binding and catalysis using a combinatorial protocol should be possible and should allow for the discovery of further catalytic peptide dendrimers with higher rate acceleration and selectivity.

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Supporting Information Available: HPLC and MS data and spectra for all monomeric and dimeric peptide dendrimers. This material is available free of charge via the Internet at http://pubs.acs.org.

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