Peptide Dendrimers as Artificial Enzymes, Receptors, and Drug-Delivery Agents

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ABSTRACT
The dendritic architecture applied to peptides provides a practical entry into globular macromolecules resembling proteins. A modular design was chosen using a divergent synthesis on solid support alternating proteinogenic α-amino acids with branching diamino acids, producing peptide dendrimers with a molecular weight of 3−5 kDa. Initial studies focused on models for hydrolases and produced esterase peptide dendrimers featuring histidine as the key catalytic residue. Variations of amino acid composition and the branching diamino acid led to enantioselective catalysts. Rate accelerations of $k_{cat}/k_{uncat} = 90\,000$ were obtained when the design was changed to monomeric peptide dendrimers alternating two amino acids with the branching unit. A combinatorial approach was developed allowing for the preparation of large libraries (>60 000 members), which were screened for B_{12} binding and catalytic activity. The peptide dendrimers were also investigated for drug delivery. Glycopeptide dendrimers conjugated to colchicine selectively inhibited the proliferation of targeted cells, whereas colchicine alone displayed high toxicity.

1. Introduction

In the late 1970s, dendrimers had emerged as a new class of compounds between small-organic molecules and polymers. The synthesis of multi-branched compounds was pioneered by Vögtle and co-workers in 1978[1] with the so-called "cascade molecules". The field was further developed in the mid-1980s when Newkome[2] synthesized tree-like molecules termed "arborols" and Tomalia[3] reported the synthesis of poly(amindoamide) and coined the term "dendrimer" [from the Greek *dendri* (tree) and *meros* (part of)] for this class of compounds. Dendrimers are monodisperse, branched macromolecules of well-defined size usually obtained by an iterative sequence of reaction steps. The most widely studied dendrimer families are the Fréchet-type polyether compositions[4] and the two now commercially available dendrimers PAMAM, poly(amidoamine), and PPI, poly(ethyleneimine).[5]

Since the first reports on dendrimers, their potential to act as catalysts and receptors has been recognized.[6] The branched structure allows for the formation of microenvironments inside the dendrimers and the placement of catalytic groups such as metal complexes at the dendritic core, branches, or the surface.[7] Dendrimers have also been investigated as enzyme models by placing enzyme cofactors at the core of organic dendrimers. Using this strategy, Diederich and co-workers introduced dendritic branches on thiazoliocyclophanes as mimics for pyruvate decarboxylate.[8a] The dendrophane promoted an aldehyde to ester oxidation by a flavin cofactor, but the reaction was 2 orders of magnitude slower than with the parent cyclophane. In another example, Breslow and co-workers reported a PAMAM dendrimer bearing a pyridoxamine at the focal point able to catalyze transamination reactions with a 1000-fold rate enhancement compared to pyridoxamine alone. Acid−base catalysis by the dendrimer amine backbone and hydrophobic substrate binding were alluded to explain the higher reactivity of the dendritic pyridoxamine.[8b]

We have approached dendrimers as protein models from a different angle, simply considering the fact that proteins are oligomers of amino acids. In nature, protein folding allows a linear polypeptide to adopt a globular conformation in which amino acid side chains are organized for function, such as the formation of a catalytic pocket. Several approaches have been used to create artificial protein mimics *de novo* from linear peptides; however, these methods are limited by the difficulty in predicting protein folding.[9] We reasoned that dendritic architectures applied to a peptide sequence could provide an efficient strategy to circumvent the protein-folding problem. A dendritic peptide[10] is topologically forced to adopt a globular shape where intramolecular interactions between amino acids are favored over intermolecular interactions leading to aggregation. In this way, protein-like structures can be created where functions such as catalysis or molecular recognition occur by constructive interactions between amino acids as in natural proteins. In this Account, we discuss our recent studies toward artificial proteins, which have focused on esterase-like dendrimers as enzyme models and on glycopeptide dendrimers for drug-delivery applications.

2. Discovery of Esterase Peptide Dendrimers

Our first peptide dendrimers were prepared by alternating proteinogenic amino acids with the symmetrical branching diamino acid **1** (Scheme 1).[11] The dendrimers were assembled by solid-phase peptide synthesis (SPPS), purified, and subsequently dimerized by disulfide bond formation using a cysteine residue placed near the dendritic core to yield peptide dendrimers with three successive layers of two amino acids at the core, four in the intermediate layer, and eight near the surface. The avail-

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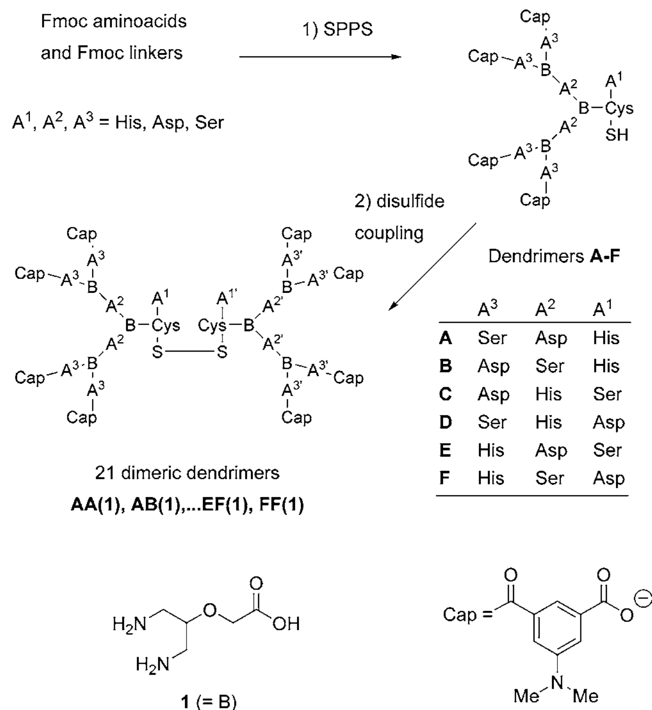
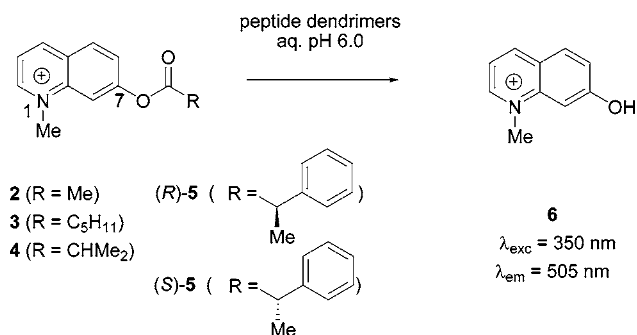
Scheme 1. Synthesis of Disulfide-Bridged Peptide Dendrimers



	A^3	A^2	A^1
A	Ser	Asp	His
B	Asp	Ser	His
C	Asp	His	Ser
D	Ser	His	Asp
E	His	Asp	Ser
F	His	Ser	Asp

Scheme 2. Fluorogenic *N*-Methyl Quinolinium Ester Substrates for Esterase Peptide Dendrimers



ability of three distinct and variable amino acid positions within this dendritic structure was used to survey combinations of aspartate, histidine, and serine, the functional amino acids present in most esterases and lipases, to obtain esterase peptide dendrimers. All 6 permutations were prepared as single dendrimers and paired to give all 21 possible dimers by disulfide bond formation. The sequences were terminated by 3-dimethylamino-isophthalamide (Cap), which was introduced as a solubilizing group and might furnish additional interactions with the substrates.

Surveying this small peptide dendrimer library for catalytic activity against a panel of fluorogenic ester substrates showed that dendrimers **EE(1)** and **EF(1)** bearing histidine residues at the surface positions catalyzed the hydrolysis of substrates **2**−**4** with saturation kinetics to produce the fluorescent quinolinium product **6** (Scheme 2). The kinetics of hydrolysis followed the Michaelis−Menten model with parameters of $K_M = 100-200$ μM and a rate acceleration up to $k_{cat}/k_{uncat} = 2400$ (Table 1). The most active dendrimers also showed a 40-fold reactivity enhancement per histidine side chain over the small molecule catalyst 4-methylimidazole. The chiral pair (*R*/*S*)-**5** was accepted as the substrate, however, without significant chiral discrimination by the dendrimers. There was no evidence for the involvement of a catalytic triad in these reactions, and the catalytic machinery seemed to consist of only histidine. Nevertheless, similar dendrimers containing histidine in combination with hydrophobic and aromatic amino acids did not show any catalytic activity.[12] As it will be seen later, the change in reactivity with the introduction of hydrophobic amino acids in the dendrimer is not confined to these examples but is a more general phenomenon.

3. Variation of the Branching Diamino Acid Leads to Enantioselective Dendrimers

One of the striking features of natural proteins is that biosynthesis allows any variation of the amino acid sequence, which enables functional optimization by mutation. For the case of synthetic peptides, SPPS is even more flexible because it can be extended to non-natural amino acids. Our first efforts to optimize our dendrimers toward higher activity and selectivity focused on diversifying the diamino acid branching unit, which was the key structure-defining element of our dendrimers. The symmetrical bis-β-alanine **7** and 2,3-diamino-propanoic acid **8** were investigated because these shorter branching units might lead to more compact structures and enforce stronger interactions between the amino acids and possibly improved catalytic properties (Figure 1).

Two series of 21 dendrimers each were prepared in analogy to the previous series (Scheme 1) using **7** or **8** as the branching unit. The resulting dendrimers behaved similarly to those derived from **1**, with dendrimers featuring surface histidine residues displaying the strongest esterolytic activity against the isobutyrate ester substrate **4**. The dendrimers derived from the elegant and symmetrical bis-β-alanine **7** were generally less active than those derived from **1**, while dendrimers derived from Dap **8** showed comparable or even higher activities than the dendrimers derived from **1** (Table 1). The best overall results were observed for the homodimer **EE(8)** with the diamino acid linker **8** on isobutyryl esters **4** with $k_{cat}/k_{uncat} = 3350$ and $K_M = 260$ μM (Table 1). However, none of the dendrimers in these two new series showed any significant enantioselectivity against the chiral pair (*R*/*S*)-**5**.

Considering the very similar properties of the dendrimers derived from the aliphatic branching units **1**−**3**, we next turned our attention to 3,5-diaminobenzoic acid **9** as a rigid branching unit (Figure 1). This group was similar to the isophthalic capping group (Cap) used in the first three series and might engage in favorable hydrophobic or π-stacking interactions with the substrate. Therefore, the dendrimers derived from **9** were simply acetylated at the N terminus. Modeling studies showed that these dendrimers were constrained in their conformation and presented a relatively open structure (Figure 2). The entire family of 6 monomeric and 21 dimeric peptide dendrimers

Table 1. Selected Kinetic Data for Esterase Peptide Dendrimers[a]

		2	**3**	**4**	(*S*)-**5**	(*R*)-**5**	E^b
	k_{uncat} (min^{-1})	3.6×10^{-4}	2.4×10^{-4}	1.2×10^{-4}	2.2×10^{-4}	2.2×10^{-4}	
4-Me Im	k_2 (mM^{-1} min^{-1})	6.7×10^{-3}	5.6×10^{-3}	4.3×10^{-3}	5.0×10^{-3}	4.8×10^{-3}	
EE(1)	K_M (mM)	0.20	0.21	0.11	0.17	0.22	
(HDS)$_2$	k_{cat} (min^{-1})	0.31	0.26	0.28	0.21	0.20	
B = **1**	k_{cat}/k_{uncat}	860	1050	2333	944	891	
	$k_{cat}/K_M/k_2$	230	220	592	244	192	1.27
EE(7)	K_M (mM)	0.25	0.07	0.69			
(HDS)$_2$	k_{cat} (min^{-1})	0.10	0.09	0.25	nd		
B = **7**	k_{cat}/k_{uncat}	268	380	2010			
	$k_{cat}/K_M/k_2$	57	226	83			
EE(8)	K_M (mM)	0.29	0.32	0.264	0.23	0.16	
(HSD)$_2$	k_{cat} (min^{-1})	0.14	0.17	0.41	0.18	0.18	
B = **8**	k_{cat}/k_{uncat}	380	690	3350	816	828	
	$k_{cat}/K_M/k_2$	70	90	360	154	243	
CC(9)	K_M (mM)	0.13	0.14	0.25	0.09	0.23	2.8
(DHS)$_2$	k_{cat} (min^{-1})	0.41	0.45	0.77	0.50	0.46	
B = **9**	k_{cat}/k_{uncat}	540	1400	4000	1740	1600	
	$k_{cat}/K_M/k_2$	470	570	720	1100	420	
AD(9)	K_M (mM)	0.17	0.12	0.54	0.10	0.07	1.0
SDH−SHD	k_{cat} (min^{-1})	0.26	0.27	0.61	0.33	0.22	
B = **9**	k_{cat}/k_{uncat}	340	840	3200	1150	760	
	$k_{cat}/K_M/k_2$	230	400	260	660	660	
MutCC(9)	K_M (mM)	0.17	0.30	0.44	0.08	0.11	1.2
(DHA)$_2$	k_{cat} (min^{-1})	0.23	0.72	0.83	0.21	0.24	
B = **9**	k_{cat}/k_{uncat}	300	2200	4300	740	830	
	$k_{cat}/K_M/k_2$	200	430	440	530	450	

[a] The structure of dendrimers is given in Figure 3. Dendrimer **E** = His(A^3), Asp(A^2), and Ser(A^1) = (HDS)$_2$. Conditions: 50−800 μM ester substrate and 5 μM dendrimer in 20 mM aqueous Bis-Tris at pH 6.0 and 25 °C. The kinetic constants given are derived from the linear double-reciprocal plots of 1/V_{net} versus 1/[S]. [b] $E = (k_{cat}/K_M((S)\text{-}\mathbf{5}))/(k_{cat}/K_M((R)\text{-}\mathbf{5}))$. nd = not determined.

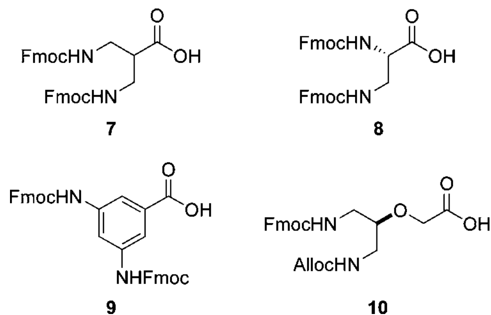


FIGURE 1. Structure of branching diamino acids **7**−**10**, in the protected form used for synthesis.

with permutations of aspartate, histidine, and serine was prepared.[13] The low reactivity of the aromatic amino group in the branching diamino acid was overcome by using symmetrical amino acid anhydrides as coupling reagents.

To our delight, dendrimers in this new library exhibited good catalytic activity and selectivity. Among the 6 monomers and 21 dimers assayed, 2 dimers with peripheral aspartate and internal histidine residues, **AD(9)** and **CC(9)** (Figure 3) showed strong catalytic activity with substrates **2**−**5**, while all of the other dendrimers were essentially inactive. Isobutyryl ester **4** was the best substrate for both dendrimers, with a maximum rate enhancement of k_{cat}/k_{uncat} = 4000 for dendrimer **CC(9)** (Table 1). The most active dendrimer **CC(9)** also exhibited significant chiral discrimination between both enantiomers of the 2-phenylpropionate (*S*)-**5** and (*R*)-**5**, with an enantiomeric ratio E = 2.8 in favor of the (*S*) enantiomer. 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 = 90 μM) compared to (*R*)-**5** (K_M = 230 μM) (Figure 4).

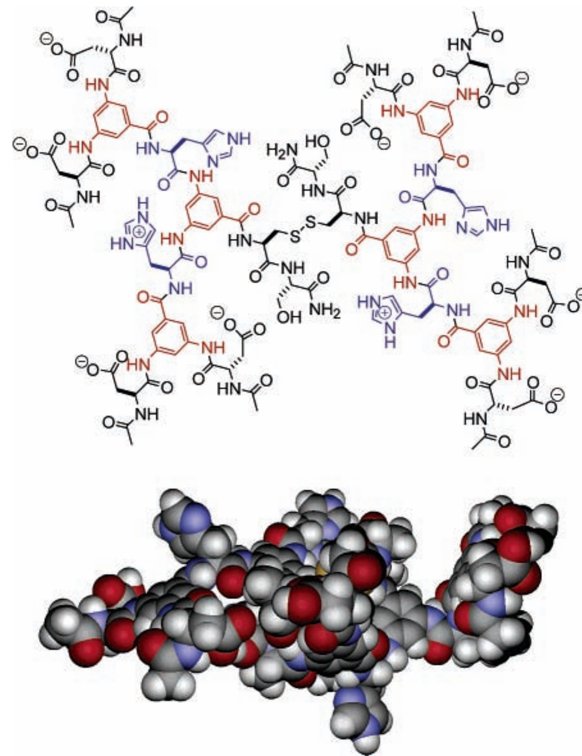


FIGURE 2. Structural formula and energy-minimized model of dendrimer **CC(9)** containing 3,5-diaminobenzoic acid **9** as a branching unit.

In the enantioselective dendrimer **CC(9)**, the catalytic histidine residues were present in four copies in the intermediate layer, an arrangement that did not show any catalytic activity in the series derived from the aliphatic branching units. This was consistent with the modeling predictions that 3,5-diaminobenzoic acid would lead to

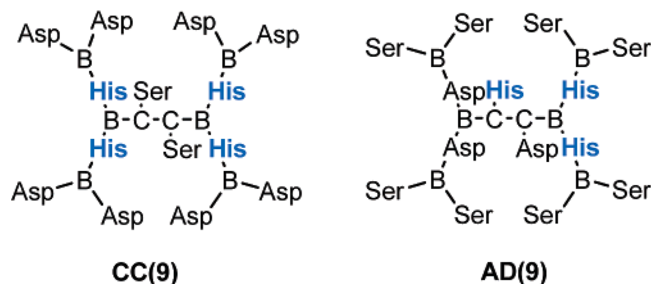


FIGURE 3. Esterase peptide dendrimers from 3,5-diaminobenzoic acid **9** as branching unit B. C = cysteine. The N termini are acetylated (kinetic data are in Table 1; the structure and cpk model of dendrimer **CC(9)** are shown in Figure 2).

dendrimers with a more extended and open structure allowing for substrate access to the dendrimer interior. The placement of catalytic residues in the dendrimer interior might also explain the significant chiral discrimination observed.

A mutational study of dendrimer **CC(9)** showed that sequential exchanges of histidine residues with alanine led to inactive dendrimers. Histidines presumably act as a nucleophilic or general-base catalytic group in the esterolysis reaction. Interestingly, exchanging a single histidine residue resulted in a 75% loss in catalytic activity, implying a cooperative effect of these residues in the dendrimer. The cooperativity effect was also evident from the fact that the nondimerized half-dendrimers with only two histidine residues were not catalytically active. In addition, the mutational experiments showed that the two serine residues at the dendrimer core could be replaced by alanine without the loss of activity, ruling out the participation of the hydroxy group in catalysis (dendrimer **MutCC(9)** in Table 1).

4. Dendrimers with a Hydrophobic Core and Functional Amino Acids at the Surface

The strongest thermodynamic driving force for protein folding is the hydrophobic collapse, whereby hydrophobic amino acids cluster together to minimize interactions with water.[14] It is also observed that hydrophobic amino acids are mostly found near the protein core, while those with polar and charged side chains occur more often at the protein–water interface. In analogy, peptide dendrimers with hydrophobic amino acids near the core and polar amino acids in the outer layers should provide interesting mimics for proteins. We prepared a series of such peptide dendrimers by using an asymmetrically protected branching unit **10** (Figure 1)[15] that allowed us to define six individually variable amino acid positions, only one of which featured a double copy at the surface (Scheme 3). The strategy was exploited to prepare a small library of 6 half-dendrimers and 21 dimers bearing a conserved hydrophobic core (constituted of phenylalanine and leucine) and a diversity of catalytic amino acids at the surface positions. These were the first examples of dendritic peptides with diverse functionalization at the periphery and belong to the very few examples of dendrimers with differentially functionalized layers.[16]

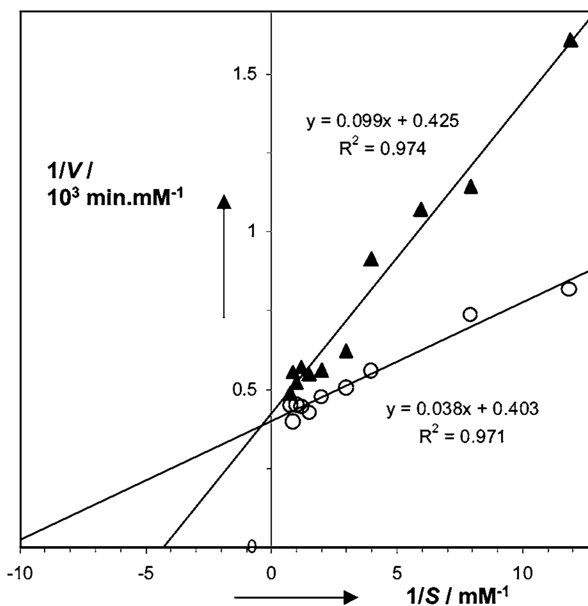


FIGURE 4. Hydrolysis of (*R*)-**5** (▲) and (*S*)-**5** (○) catalyzed by dendrimer **CC(9)**. Conditions: see footnote *a* of Table 1.

In contrast to the previous series, these hydrophobic core dendrimers did not hydrolyze 7-hydroxy-*N*-methylquinolinium esters **2**–**5**. Further screening showed that several dendrimers in this series catalyzed the hydrolysis of hydroxypyrene trisulfonate esters (Scheme 4). The most active dendrimer was the homodimer **LL** with the peripheral arrangement (His–His–Ser–Asp)$_2$, with k_{cat} = 0.045 min^{-1}, K_M = 120 μM, and k_{cat}/k_{uncat} = 1000 for the butyrate ester **12** measured at pH 6.0, corresponding to a 500-fold reactivity enhancement relative to the monomeric catalyst 4-methylimidazole ($k_{cat}/K_M/k_2$ = 470). The catalytic efficiency increased 4-fold between the acetate **11** ($k_{cat}/K_M/k_2$ = 380) and the nonanoate **13** ($k_{cat}/K_M/k_2$ = 1600), because of a 10-fold stronger binding of the longer chain ester. These results provided evidence for the interaction of the substrate with the hydrophobic core of the dendrimer. The monomeric dendrimer **L** showed similar activity in this case but with higher K_M values (k_{cat} = 0.046 min^{-1}, K_M = 190 μM, k_{cat}/k_{uncat} = 1100, and $k_{cat}/K_M/k_2$ = 300 for the butyrate ester **12**), suggesting that dendrimer size primarily influenced binding. The possibility of enantioselective catalysis was not investigated in the present series. In fact, we simultaneously discovered a new type of peptide dendrimers built around a different topology and exhibiting higher catalytic activities. These became the focus of our investigations as described below.

5. A Strong Positive Dendritic Effect in Esterase Dendrimers

The peptide dendrimers discussed thus far were obtained in a sequence alternating an amino acid (A) with a branching diamino acid (B), corresponding to iterations of an A_2B dendron. Our next synthetic explorations were directed at peptide dendrimers featuring two amino acids rather than one in each branch, corresponding to a dendron of type $(A^2A^1)_2B$. The synthesis of these new dendrimers was surprisingly easy. Third generation den-

Scheme 3. Synthesis of Peptide Dendrimers with Differentially Functionalized Surface Residues around a Hydrophobic Core[a]

	A^3	A^5	A^6
H	Ser	His	Asp
I	Ser	Asp	His
J	Asp	Ser	His
K	Asp	His	Ser
L	His	Ser	Asp
M	His	Asp	Ser

6 monomers **H-M**

21 dimers **HH, HI,...LM, MM**

[a] Conditions: Deprotecting steps: i = DMF, piperidine (4:1); ii = $PhSiH_3$ (25 equiv), CH_2Cl_2 for 3 min, and then $Pd(PPh_3)_4$ (0.1 equiv). Coupling steps: iii = BOP/DIEA. Capping steps: iv = Ac_2O/CH_2Cl_2 (1:1). (1) i and then iii with FmocHisOH; (2) i and then iii with FmocCys(Trt)OH; (3) i and then iii with **10**; (4) i and then iii with FmocPheOH; (5) i and then iii with **1**; (6) i and then iii with $FmocA^3OH$; (7) i and then iv; (8) ii and then iii with FmocLeuOH; (9) i and then iii with **10**; (10) i and then iii with $FmocA^5OH$; (11) i and then iv; (12) ii and then iii with $FmocA^6OH$; (13) i and then iv; (14) TFA cleavage and HPLC purification; and (15) disulfide coupling with 2,2′-dithiopyridine.

drimers could be obtained in substantially higher yields than the A_2B-based dimers. Dendrimers of increasing generation numbers using a $(His\text{-}Ser)_2Dap$ dendron were then prepared to explore their possible esterolytic activity.[17] The synthesis succeeded up to the fourth generation dendrimer with 16 surface groups, providing a regular series of increasing size **G1**−**G4** (Figure 5).

Dendrimers **G1**−**G4** accepted the acyloxypyrene trisulfonate as substrates, similarly to the hydrophobic core dendrimers described above. However, the catalytic activities were considerably higher, particularly in the higher generation dendrimers **G4**, which had a rate acceleration over the background of k_{cat}/k_{uncat} = 39 000 for the hydrolysis of **12**, corresponding to a 1200-fold reactivity increase per histidine side chain by a comparison of the specificity constant k_{cat}/K_M to the reference catalyst 4-methylimidazole (Table 2).

Scheme 4. Peptide Dendrimer Catalyzed Hydrolysis of Acyloxypyrene Trisulfonates

dendrimers (2.5 mol%) aq. pH 5.5-6.5; RCO_2H

11 (R = CH_3)
12 (R = C_3H_7)
13 (R = C_8H_{17})
14 (X = OH)

The kinetic data showed a strong positive dendritic effect on catalytic efficiency (Figure 6). This effect was surprising because dendritic systems with peripheral catalytic groups usually exhibit a weaker catalytic activity per catalytic group compared to the monomeric catalyst. In fact, a negative effect of dendrimer size on catalysis must be expected because of steric crowding, unless additional effects increasing catalytic efficiency can be created in larger dendrimers. In our case, the proportionality of k_{cat} to dendrimer size suggested either no steric crowding in larger dendrimers or the occurrence of a cooperative effect, increasing catalysis and compensating for steric crowding; for example, a lower pK_a of the histidine side-chain increases the concentration of free base.

The proportionality of substrate binding $1/K_M$ to dendrimer size, participating in the positive dendritic effect

FIGURE 5. Peptide dendrimers **G1**−**G4** with iterations of a $(His\text{-}Ser)_2Dap$ dendron.

Table 2. Kinetic Parameters for Esterase Dendrimers G1–G4 Measured at pH 5.5[a]

		G1	G2	G3	G4
11	K_M (μM)	840	110	41	35
	k_{cat} (min^{-1})	0.080	0.16	0.30	0.86
	k_{cat}/k_{uncat}[b]	1800	3600	6800	20 000
	$(k_{cat}/K_M)/k_2$[c]	140	2000	10 500	35 000
	$(k_{cat}/K_M)/k_2$/His	46	280	700	1100
12	K_M (μM)	450	140	63	29
	k_{cat} (min^{-1})	0.031	0.11	0.24	0.55
	k_{cat}/k_{uncat}[b]	2200	8000	17 000	39 000
	$(k_{cat}/K_M)/k_2$[c]	140	1600	7900	38 000
	$(k_{cat}/K_M)/k_2$/His	48	230	530	1200
13	K_M (μM)	1600	67	13	5.8
	k_{cat} (min^{-1})	0.099	0.096	0.15	0.39
	k_{cat}/k_{uncat}[b]	4500	4400	6700	18 000
	$(k_{cat}/K_M)/k_2$[c]	130	3000	23 000	140 000
	$(k_{cat}/K_M)/k_2$/His	44	420	1600	4500

[a] Conditions: 5 mM aqueous citrate at pH 5.5 and 27 °C. [b] k_{uncat} (min^{-1}) = 4.4 × 10^{-5} (**11**), 1.4 × 10^{-5} (**12**), and 2.2 × 10^{-5} (**13**). [c] k_2(4-Me-Im) (mM^{-1} min^{-1}) = 7.0 × 10^{-4} (**11**), 4.9 × 10^{-4} (**12**), and 4.8 × 10^{-4} (**13**).

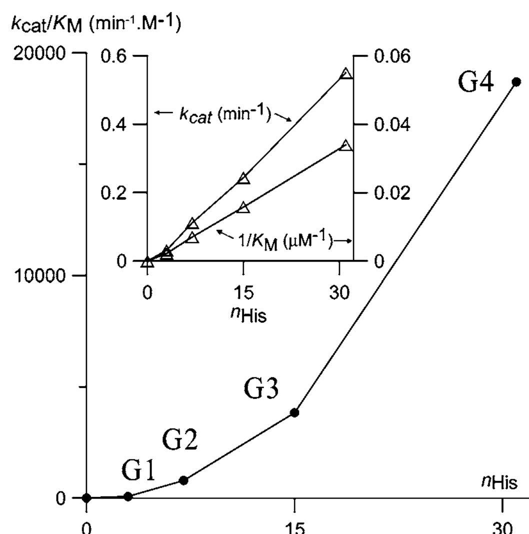


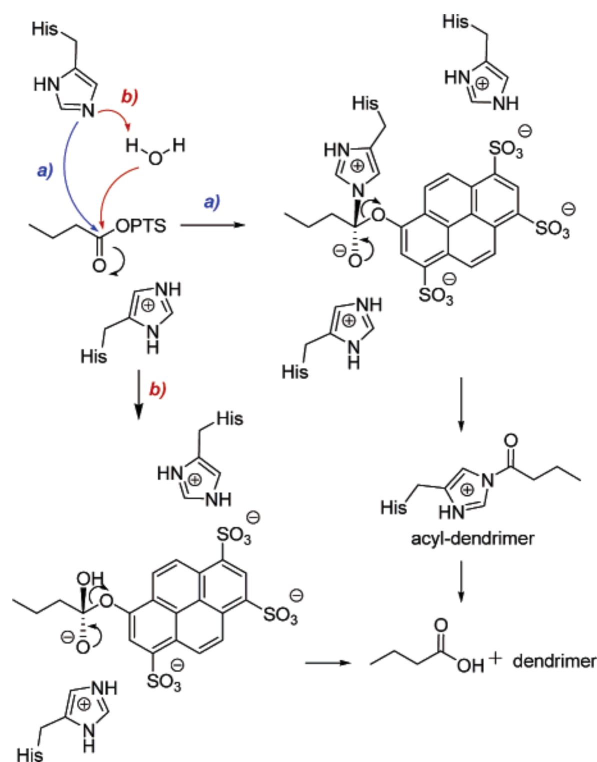
FIGURE 6. Positive dendritic effect on catalytic proficiency k_{cat}/K_M in the hydrolysis of butyrolxypyrene trisulfonate substrate **12** catalyzed by peptide dendrimers **G1–G4**.

observed, was traced back to the increasing binding to the acyl chain of the substrates in higher generation dendrimers. Indeed, the reaction product hydroxypyrene trisulfonate **14** bound all four dendrimers **G1–G4** equally with K_D = 330 μM, an approximately 10-time weaker binding compared to the substrates.

6. Reaction Mechanism of Esterase Peptide Dendrimers

The small molecule catalyst 4-methylimidazole catalyzes the esterolytic reactions of the quinolinium and pyrene trisulfonate ester substrates of our dendrimers. No intermediates can be detected during hydrolysis, implying either that 4-methylimidazole acts as a general-base catalyst for the activation of water as a nucleophile or that a putative acyl–imidazole intermediate is hydrolyzed faster than it is formed. A similar role should be played by one or several histidine side chains in the esterase peptide dendrimers. The absence of a burst phase in the time profile of product formation suggests that an acyl–dendrimer intermediate is either not formed (general base) or hydrolyzed faster than it is formed. The pH-rate profile of the catalytic rate constant k_{cat} for the dendrimer-catalyzed reactions is flat between pH 5.0 and 7.0. This behavior hints at bifunctional catalysis by two histidine side chains, one acting in the free-base form as the nucleophilic or general-base catalyst and another acting in the protonated form, for example, in stabilizing the oxyanion intermediate (Scheme 5).

Scheme 5. Mechanistic Proposal for Dendrimer-Catalyzed Ester Hydrolysis[a]



[a] (a) Nucleophilic attack of histidine generated an acyl–dendrimer intermediate. (b) General-base-catalyzed nucleophilic attack of a water molecule.

The involvement of histidine residues in dendrimer activity was shown by extensive histidine-to-alanine replacement in dendrimer **G3**, which resulted in the complete loss of catalytic activity. In contrast, the corresponding serine-to-alanine exchange had only a marginal effect on reaction rates, showing that the hydroxyl group is not directly involved in catalysis. Corroboration of histidine participation in catalysis was obtained by an extensive mutational study of dendrimer **G3**, in which we prepared and characterized 33 different dendrimer mutants with the help of an automated synthesis protocol.[18] In the course of this study, we also discovered that extensive serine-to-threonine replacement provides a dendrimer with a 10-fold higher activity, with rate acceleration k_{cat}/k_{uncat} = 90 000 and substrate binding K_M = 160 μM for butyrate ester **12**, which corresponds to an almost 20 000-fold rate acceleration per catalytic site compared to 4-methylimidazole as the reference catalyst (Figure 7). This dendrimer is the most efficient catalytic peptide dendrimer that we have found to date.

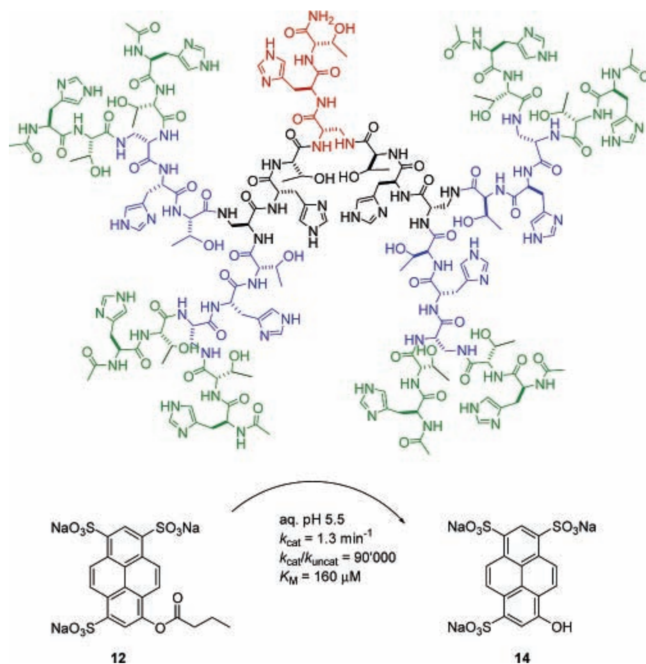


FIGURE 7. Best esterase peptide dendrimer to date with a 10^5-fold rate enhancement.

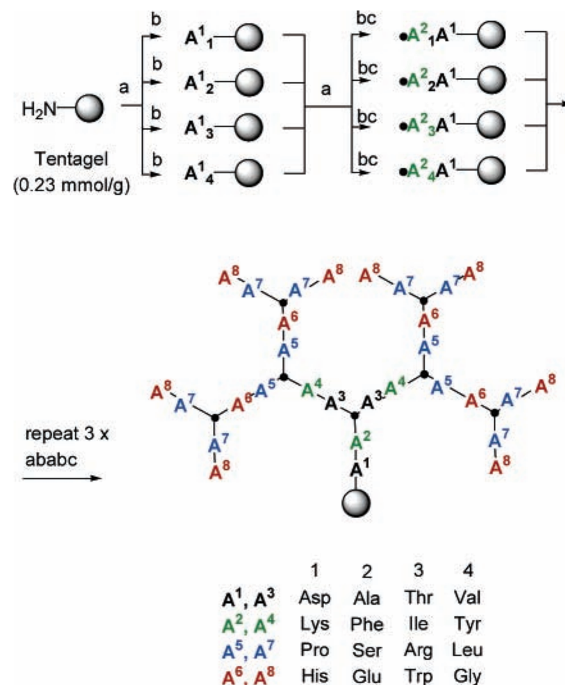


FIGURE 8. Combinatorial split-and-mix synthesis of peptide dendrimers. (●) L-2,3-diaminopropanoic acid (Dap). Using 4 amino acids/variable position, A^i (i = 1−8) gives 4^8 = 65 536 members. Conditions: (a) suspend the whole resin batch in DMF/DCM (2:1, v/v), mix via nitrogen bubbling for 15 min, and split the batch in four equal portions 1−4; (b) in each portion x = 1−4: Fmoc-A^n_x-OH, PyBOP, DIEA, DMF, generation × 60 min, and then DMF/piperidine (4:1, v/v), 2 × 10 min; (c) same as b with Fmoc-Dap(Fmoc)-OH.

7. Combinatorial Libraries of Peptide Dendrimers

The strength of peptide dendrimers as artificial protein models lies in the combination of a simple dendritic design for the structure with a robust synthetic protocol provided by well-established SPPS. A large number of amino acid building blocks are currently commercially available for SPPS, which opens the possibility to prepare large numbers of different peptide dendrimers. Although we were quite successful in discovering active dendrimers in a small series of 20−30 different peptide dendrimers prepared individually, we were interested in exploiting the potential of split-and-mix combinatorial peptide synthesis[19] to prepare libraries containing thousands of different dendrimer sequences on solid support. In split-and-mix synthesis, the polymer support used for SPPS is mixed and divided again in portions to combine the different building blocks assigned to each position in any peptide sequence. Although this protocol generates all possible combinations of building blocks, any individual bead of the solid support contains only a single sequence because it reacted with only one building block at each of the coupling steps. The method has been used to prepare libraries of linear peptides for identification of sequences with different functions. Examples for catalysis include the combinatorial undecapeptide library described by Berkessel[20] containing 625 peptides, which led to the discovery of peptide−zirconium complexes that mediated phosphate hydrolysis in aqueous solution. In another approach, fluorescently labeled split-and-mix peptide libraries were applied to the discovery of octapeptide catalysts for enantioselective acyl transfer reactions.[21] The library of 100 000 octapeptides incorporating a histidine residue revealed catalysts for kinetic resolution of secondary alcohols with a selectivity factor of up to 50 in toluene.

We have realized a combinatorial synthesis of peptide dendrimers using four different building blocks at each of the variable amino acid positions, which resulted in a library of 65 536 different dendrimers for a third generation peptide dendrimer with two variable amino acids per branch (Figure 8).[22]

We have used this combinatorial protocol to discover active peptide dendrimers for both catalysis and binding by direct screening for the function on the polymer beads. Beads carrying catalytically active esterase peptide dendrimers were identified by soaking a library sample with an aqueous solution of the fluorogenic substrate **12** and observing the formation of the fluorescent products under the microscope (Figure 9). The sequence of the dendrimers on these fluorescent beads was determined by picking the beads and submitting them to amino acid composition analysis, which allows for unequivocal sequence assignment in the dendritic design. The active dendrimers showed a strong consensus for the presence of histidine residues at the surface positions (Table 3). When the peptide dendrimers were resynthesized, they exhibited the expected esterase activity on substrate **12** and its analogues, with catalytic parameters in the higher range of our observations (k_{cat} = 0.13 min^{-1}, K_M = 210 μM, k_{cat}/k_{uncat} = 10 000, and $k_{cat}/K_M/k_2$ = 1500 for the butyrate ester **12**) (Figure 10).

The combinatorial library approach also allowed us to discover peptide dendrimers binding to vitamin B_{12}.[22a] Soaking of the combinatorial library with an aqueous

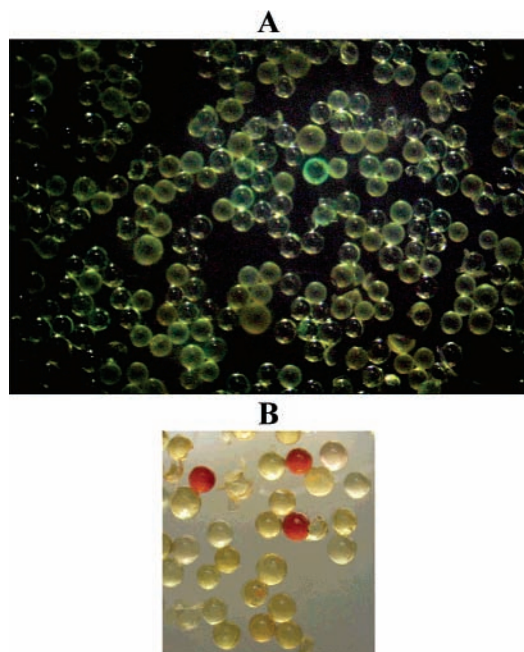


FIGURE 9. Screening of the catalytic peptide dendrimer library. (A) Microscope picture under illumination with UV 356 nm. Beads were soaked with 80 μM 8-butyryloxypyrene-1,3,6-trisulfonate **12** in aqueous 20 mM Bis-Tris at pH 6.0 and spread out in a Petri dish. The green bead near the center contains a catalytic sequence. (B) Screening for binding to vitamin B_{12}. Conditions: 30 min of equilibration in aqueous PBS (10 mM phosphate and 160 mM NaCl at pH 7.4) containing 400 μM cyanocobalamin (vitamin B_{12}), followed by washing with PBS and water.

Table 3. Peptide Dendrimer Sequences Identified by Amino Acid Analysis of Active Beads from the Combinatorial Library and the Corresponding Consensus Sequences[a]

	number	A^8	A^7	A^6	A^5	A^4	A^3	A^2	A^1
hits for hydrolysis of 8-butyryloxy pyrene-1,3,6-trisulfonate **12**[b]	1	W	**S**	***G***	R	**K**	**V**	**I**	A
	2	**H**	L	**H**	***S***	Y	A	**I**	D
	3	**H**	L	***G***	**L**	Y	T	**I**	**V**
	4	**H**	P	***G***	P	**K**	T	**I**	A
	5	E	R	***G***	***S***	***I***	**V**	**I**	**V**
	6	G	R	W	R	***I***	**V**	**I**	A
	7	**H**	**S**	**H**	**L**	F	A	F	D
	8	**H**	**S**	***G***	R	***I***	A	**I**	**V**
	9	**H**	**S**	**H**	P	**K**	**V**	F	**V**
	10	G	R	***G***	P	***I***	**V**	**I**	**V**
consensus sequences	**C11**	**H**	**S**	**H**	**L**	**K**	**V**	**I**	**V**
	C12	**H**	**S**	**G**	***S***	***I***	**V**	**I**	**V**
hits for binding to vitamin B_{12}[c]	13	**W**	L	H	***S***	***I***	A	***K***	A
	14	E	**P**	**G**	**R**	**Y**	**T**	**Y**	**D**
	15	W	**P**	***E***	***S***	**Y**	A	**Y**	**D**
	16	***G***	**P**	W	P	**Y**	**V**	***K***	**V**
	17	***G***	***R***	***E***	**R**	***I***	***T***	I	**D**
	18	H	L	**G**	**R**	K	**V**	***K***	**D**
	19	**W**	***R***	***E***	***S***	***I***	**V**	I	***V***
consensus sequences	**C20**	**W**	**P**	**G**	**R**	**Y**	**V**	**Y**	**D**
	C21	***G***	***R***	***E***	***S***	***I***	***T***	***K***	***V***

[a] Dendrimers marked bold were resynthesized by Fmoc-SPPS and purified. [b] All N termini were N-acetylated. Screening conditions: Beads soaked in 20 mM aqueous Bis-Tris buffer containing 80 μM 8-butyryloxypyrene-1,3,6-trisulfonate at 25 °C for 30 min. Hits are green-fluorescent under UV 356 nm. [c] N termini are free amine. Screening conditions: 30 min of equilibration in aqueous PBS (10 mM phosphate and 160 mM NaCl at pH 7.4) containing 400 μM cyanocobalamin (vitamin B_{12}), followed by washing with PBS and water. Hits are bright orange.

solution of cyanocobalamin resulted in very few red-colored beads, indicating binding (Figure 9). Sequences for B_{12} binding showed predominantly tryptophan and glycine at position A^8 (Table 3). Several of these dendrimers were resynthesized and purified, and the soluble dendrimers exhibited the desired binding to vitamin B_{12}, however, relatively weakly (K_D > 100 μM). A follow-up study using focused libraries led to the identification of several dendrimers with micromolar binding, as will be reported in due course.

Further studies of both catalytic and binding peptide dendrimers based on combinatorial libraries are currently ongoing. For example, we have discovered aldolase peptide dendrimers by screening libraries biased toward aldolase activity by using lysine and proline residues either at the dendrimer core or at surface positions. These dendrimers exhibit strong activity in water and good enantioselectivity.

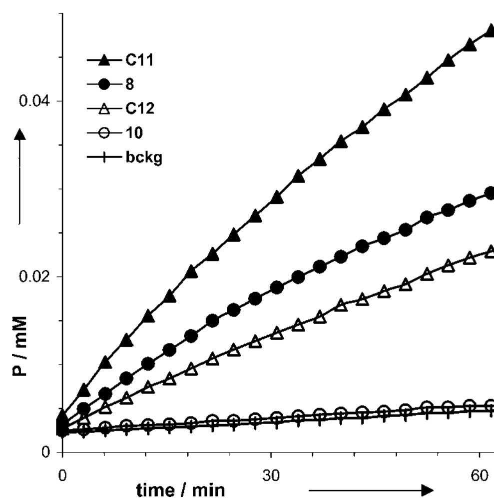


FIGURE 10. Time course of hydrolysis of 8-butyryloxypyrene-1,3,6-trisulfonate **12** catalyzed by peptide dendrimers from the combinatorial library. Conditions: 200 μM substrate, 5 μM dendrimer, and aqueous 20 mM Bis-Tris at pH 6.0 and 25 °C.

8. Glycopeptide Dendrimers for Drug Delivery

The peptide dendrimer chemistry developed to study artificial enzyme design might also be useful in the area of biomedicine when applied for molecular recognition and targeting rather than catalysis. Indeed, synthetic peptides can be produced economically for use as drugs, and peptide dendrimers could be accessible to production by the same methods. In that perspective, we have investigated our peptide dendrimers as drug-delivery agents. A number of recent studies have shown that dendrimers can be used to deliver drugs to tumor cells. Most current systems are based on non-natural dendrimers, such as PAMAM, and carry multiple copies of the drugs together with a targeting device.[23]

We have focused our attention on using the dendrimer as carrier of a single drug molecule placed at the core while functionalizing the dendrimer surface with carbohydrates to favor cellular uptake.[24] The first drug studied was colchicine, a natural product that is not employed for chemotherapy because of its cytotoxicity and would

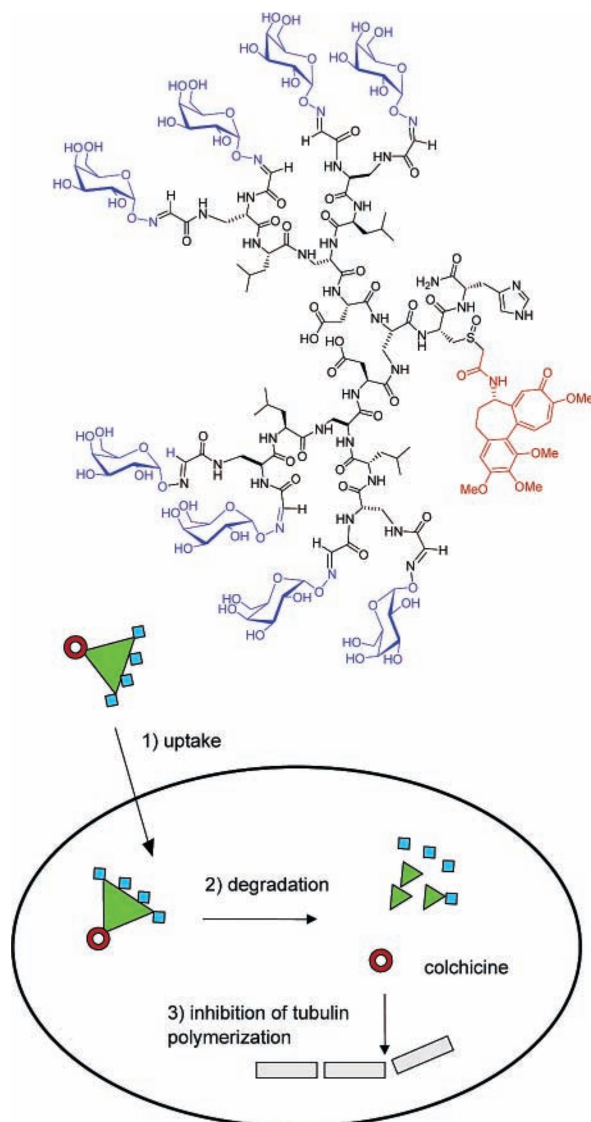


FIGURE 11. Glycopeptide dendrimer drug conjugates deliver colchicine selectively to cancer cells.

benefit from a vehicle capable of targeted drug delivery. Our results show that glycosylated peptide dendrimers loaded with a colchicine molecule at their core are cytotoxic to cancer cell lines such as HeLa cells and are more selective toward these cancer cells than to noncancerous cells compared to colchicine itself (Figure 11). The mechanism of action of these conjugates probably involves uptake and intracellular degradation by proteolysis to release the active drug. The ability of gylcodendrimers to act as drug-delivery agents has recently been confirmed using other dendrimer constructs and different cell lines, also using drugs other than colchicine. While our initial constructs were only active in the micromolar range, we recently found dendrimer drug conjugates active in the nanomolar range as well.

9. Conclusions

In studying peptide dendrimers, our goal is to produce dendritic artificial proteins that can be active in aqueous medium. The present Account reviewed our initial studies, which have been mostly directed at esterase enzyme mimics. These studies showed that enzyme-like catalysis with substrate binding and turnover can be found in dendritic peptides. The catalytic properties strongly depend upon the amino acid composition and sequence of the dendrimers, with effects similar to those observed with proteins, such as hydrophobic substrate binding and cooperative effects between side chains to increase catalysis.

Our combinatorial protocol for synthesis of large libraries of peptide dendrimers opens the way for discovering functions that are not readily accessible by design. Nevertheless, the combinatorial approach can in the best case only be used to support a rational design idea, which is expressed by selecting the placement of critical amino acids at predefined positions within the dendrimer. A more reliable design should be based on a dendrimer crystal structure; however, our efforts in that direction have not succeeded to date. The difficulty in obtaining crystals might be caused by the structural flexibility of the dendrimers. Structural flexibility became in fact evident when peptide dendrimers were investigated by molecular dynamics simulation, the method that we are currently using to design peptide dendrimer topologies that fold into compact three-dimensional structures. We are also exploring strategies to prepare structurally restrained peptide dendrimers, including the use of rigid branching diamino acids and the formation of intramolecular disulfide bridges.

The prospect of creating synthetic dendritic miniproteins, with all of the possibilities given by the use of non-natural amino acids, is a fascinating perspective from a fundamental point of view because it provides a counterpart to the linear assembly selected by nature. This approach to artificial proteins also has real potential to deliver practical products given the reliability of SPPS even on an industrial scale. Such products might be particularly relevant in the field of biomedicine, for example, for the development of targeted drug delivery as with the glycopeptide dendrimers discussed above.

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