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Proteolysis of Peptide Dendrimers

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The ability of proteins and peptides to undergo proteolysis is essential to their biological function. Herein, we report the first detailed study of the protease reactivity of peptide dendrimers. Dendrimers are regularly ramified, tree-like synthetic macromolecules with promising application in technology and medicine. Using trypsin and α-chymotrypsin cleavage sites as models, we show that the protease reactivity of peptide dendrimers can be controlled by the degree of branching. Dendrimers with two or three amino acids between branching points were readily cleaved by trypsin irrespective of the position of the reactive sequence within the dendrimers, for example in **D1**, $(Ac\text{-}Gly\text{-}Phe\text{-}Pro)_4(Dap\text{-}Hyp\text{-}Arg^{\downarrow}Met)_2Dap\text{-}Ser\text{-}Gly\text{-}\beta Ala\text{-}NH_2$, and **D12**, $(Ac\text{-}Ser\text{-}Ala)_8(Dap\text{-}Ala\text{-}Arg^{\downarrow})_4(Dap\text{-}Ala\text{-}Asp)_2Dap\text{-}Phe\text{-}Ala\text{-}Lys^*\text{-}NH_2$ (Dap: (*S*)-2,3-diaminopropionic acid branching point, Hyp: hydroxyproline, Lys*: FITC-labeled lysine, $^{\downarrow}$: cleavage site). On the other hand cleavage was blocked in more compact dendrimers with only one amino acid between branching points, for example in **D18B**, $(Ac\text{-}Glu)_8(Dap\text{-}Phe)_4(Dap\text{-}Arg)_2Dap\text{-}Leu\text{-}NH_2$). The control of proteolysis by topology provides a novel possibility to tune the biological properties of peptide dendrimers not available in linear peptides, and should be generally useful for their use as functional biomolecule analogues, for example, in the context of drug delivery applications.

Introduction

The ability of natural proteins and peptides to undergo proteolysis is essential to their biological function, and allows phenomena such as activation of proenzymes and prohormones, the removal of leader sequences controlling cellular localization, or the elimination of unwanted or dysfunctional products.[1] In synthetic peptides proteolytic reactivity can be exploited to add function, in particular in targeted prodrug activation strategies.[2] The proteolysis of any peptide or protein depends on its amino acid sequence. All proteases indeed exhibit a certain degree of selectivity, in particular for the amino acids adjacent to the cleavage site.[3] Proteolysis also depends on the three dimensional structure, whereby exposed loops at protein surfaces are usually reactive, while tightly folded domains are protected.[4]

Herein, we report on the protease reactivity of peptide dendrimers. Dendrimers are synthetic macromolecules with a ramified, tree-like topology rarely found in natural macromolecules.[5] This topology enforces a globular shape enabling properties such as microenvironment effects at the dendritic core, which are useful for encapsulation,[6] multivalency effects at the dendrimer surface that can be exploited in binding,[7] and catalysis.[8] Dendrimers assembled from amino acid building blocks are particularly attractive as models for proteins, such as enzymes and glycoproteins.[9,10–13] Structurally, peptide dendrimers behave as molten globules in aqueous solution.[14] Contrary to folded proteins, however, they cannot be denatured to a linear disordered state; this suggests that they might be resistant to proteolytic degradation by blocking access of the protease to possible cleavage sites. Indeed multiple antigenic peptides, which are synthetic linear peptides attached to a dendritic polylysine core, have been reported to be resistant to proteolytic degradation.[15] A detailed quantitative study of dendrimer proteolysis was, however, not carried out.

Using trypsin and chymotrypsin reactive sequence motives as models, we show that the proteolytic reactivity of peptide dendrimers can be controlled by the frequency of branching points. Peptide dendrimers with two or three amino acids between the branching points, such as **D1**, **D11** or **D12**, display only limited hindrance to proteolysis. On the other hand trypsin and chymotrypsin cleavage sites placed within more compact dendrimers with only one amino acid between branching points, such as **D15B**, **D18B** and **D19B**, show significant and sometimes complete resistance to proteolysis while their linear peptide analogues are rapidly cleaved (Figure 1). The control of proteolysis by topology provides a novel possibility to tune the biological properties of peptide dendrimers not available in linear peptides, and should be generally useful for their use as functional biomolecule analogues, for example, in the context of drug delivery applications.[16]

Results and Discussion

Both literature precedents and chemical intuition suggest that peptide dendrimers should not react with proteases due to steric hindrance from branching at the cleavage site. We set out to probe the protease reactivity of peptide dendrimers

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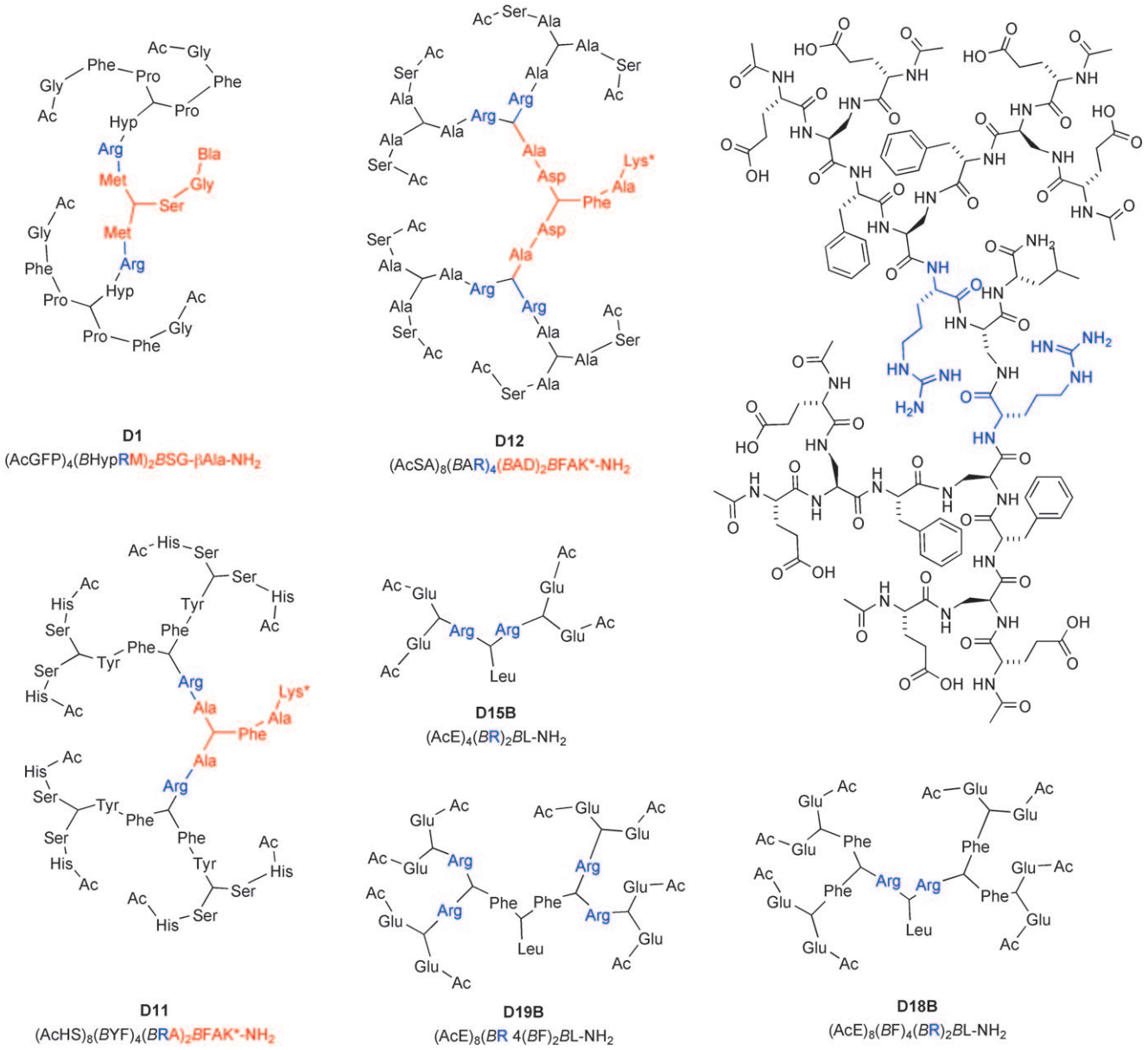

Figure 1. Examples of peptide dendrimers found to be either reactive (**D1**, **D11**, **D12**) or resistant (**D15B**, **D18B**, **D19B**) to trypsin cleavage. All branching points are (*S*)-2,3-diaminopropionic acid (Dap, *B*) and the C-termini are $CONH_2$; K* is side-chain FITC-labeled lysine. For dendrimer **D18B** the full structure is also shown; see also Tables 1 and 2.

using a solid-supported peptide dendrimer combinatorial library[17] and our simple on-bead protease profiling assay recently reported for linear peptides.[18] In this assay the free amino termini unmasked by proteolysis are derivatized by reductive alkylation with the dye-labeled aldehyde **1**, which reveals proteolysis on the surface of the solid support known as "bead-shaving".[19] The stained beads are then analyzed by total hydrolysis and amino acid composition analysis, which allows the determination of the amino acid sequence.[20] We focused on second-generation peptide dendrimers containing three amino acids per branch, which might be conformationally sufficiently flexible to allow access of a protease to a cleavage site within the branches. A 4096-member combinatorial library was prepared with variable amino acid positions in the first and second generation tripeptide branches following our previously reported library design protocol (Figure 2).[17]

While no reaction could be detected with trypsin or α-chymotrypsin under the conditions of protease profiling with linear peptides (25 °C, 1 h in aqueous buffer),[18] incubation at 36 °C in the presence of dimethylsulfoxide (20%, *v/v*) as cosolvent provided a significant proportion of stained beads after reductive alkylation. In both cases, the sequences of protease reactive (stained) and unreactive (unstained) beads were analyzed (Table S1–S5 in the Supporting Information). All trypsin-reactive dendrimers contained at least one arginine, as expected from the cleavage selectivity of this enzyme,[3] and were also strongly enriched in threonine at position X^7 and methionine at X^3. By contrast unreactive sequences were associated

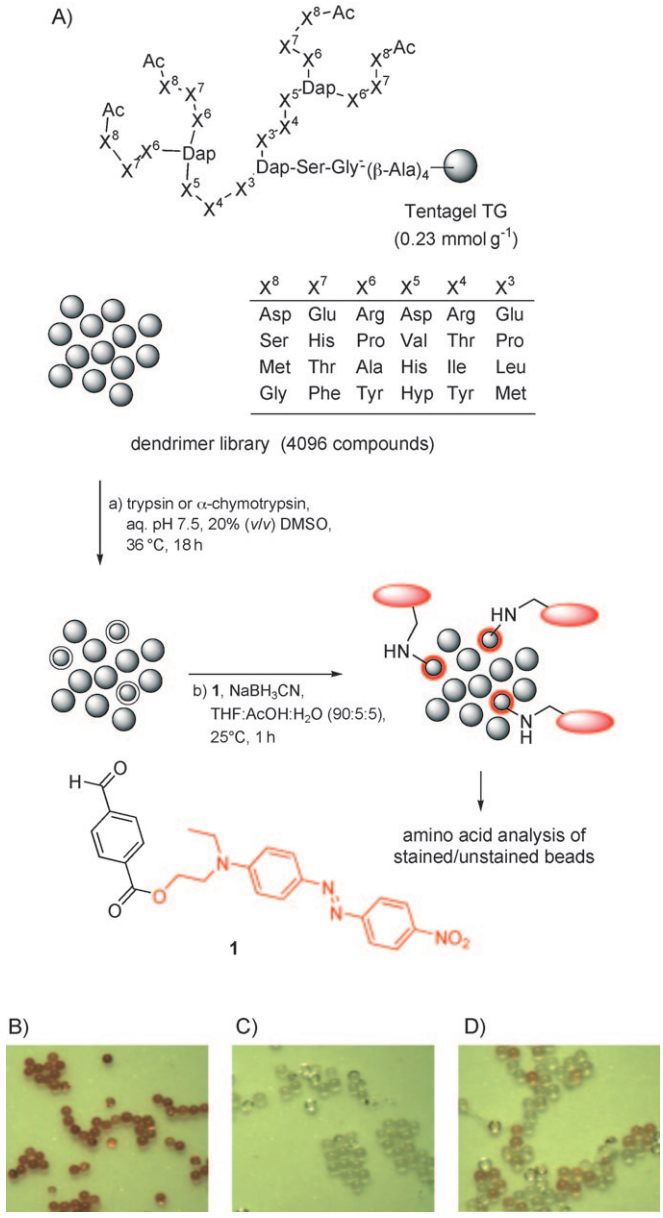

Figure 2. A) On-bead proteolysis assay of a peptide dendrimer combinatorial library. Beads showing: B) the positive control: nonacetylated library; C) negative control: unreacted, acetylated library; D) assay: trypsin treated acetylated library.

with anionic residues (Asp in X^8 or and Glu in X^7), tyrosine at X^6 or X^4, and proline at X^3. For α-chymotrypsin, which cleaves at aromatic and hydrophobic amino acids, the reactive sequences indeed preferentially contained Met at X^8/X^3, Phe at X^7, and Leu at X^3. A strong consensus was also observed for arginine at X^6 and X^4, which might be explained by an electrostatic interaction between the cationic sequences and the anionic α-chymotrypsin, the isoelectric point of which is at pH 5.4.[21] On the other hand α-chymotrypsin unreactive beads generally indicated a prevalence of anionic residues (Glu at X^7/X^3 and Asp at X^8/X^5) as well as Ala and Pro at X^6, including some Phe–Pro sequences, which are known to be resistant to cleavage by α-chymotrypsin.

Protease reactive dendrimer sequences **D1**–**D7** were selected from the solid-supported assay and synthesized by SPPS, cleaved from the support, and purified by RP-HPLC. We also prepared two anionic, trypsin-unreactive sequences from the library, **D8** and **D9**, in which an arginine was introduced artificially to produce a tryptic cleavage site. The control linear sequences **L1**–**L9** were also prepared by replacing the branching (*S*)-2,3-diaminopropanoic acid (Dap) residues by alanine (Table 1).

The solution assays showed that the dendrimers identified as either protease reactive or protease resistant exhibited the corresponding reactivity in solution; this result confirmed the on-bead assay. The designed, anionic tryptic sequences, **D8** and **D9**, also underwent cleavage but with a much lower reactivity, which is in agreement with the fact that they were not identified in the solid-supported assay. The linear peptide analogues of the dendrimers **L1**–**L9** were generally 3–5-times more reactive towards the proteases than the corresponding dendrimers. The limited yet significant reduction in reactivity for the dendrimers is probably caused by the reduced accessibility of the cleavage sites induced by branching. This trend was inverted in the case of the very weakly reactive dendrimer **D9** and its linear analogue **L9** upon cleavage at high trypsin concentration; this shows that in this case, as for the unreactive sequences **D3**/**L3**, **D4**/**L4** and **D7**/**L7**, the lack of reactivity was caused by the amino acid sequence and not by dendritic branching.

The experiments above indicated that the amino acid sequence rather than the dendritic branching controlled reactivity in dendrimers derived from the combinatorial library. The dendritic topology itself generally reduced reactivity in most cases, but did not block reactivity. This observation raised the question whether it might be possible to take a protease reactive peptide sequence and "hide" it from proteolysis by dendritic branching, for instance, by using a more compact dendrimer design with fewer amino acids between branching points.

In a first attempt towards this goal, we prepared the third generation peptide dendrimers **D10**–**D12** with only two amino acids per branch and trypsin or α-chymotrypsin cleavage sites at various positions within the branches. A fluorescence label was introduced at the dendritic core to facilitate analysis of the fragments. We have shown previously with the example of esterase-active dendrimers that this type of third generation dendrimer can be hydrophobically collapsed, and that their degree of compaction is comparable to molten globule, and sometimes, fully-folded proteins; this suggests that such hydrophobically collapsed dendrimers might be resistant to proteolysis.[14] However, while these dendrimers were much less reactive than the second generation dendrimers above, quantitative proteolysis could be obtained when using 50 $\mu g\,mL^{-1}$ of either trypsin or α-chymotrypsin. Specific cleavage was confirmed in the case of trypsin by identification of the fragments by MS, which showed that all arginine cleavage sites had reacted (Table 2).

The decrease in proteolytic reactivity obtained by reducing the branch length from three to two amino acids suggested that even more compact dendrimers might become entirely

Table 1. Synthesis and proteolytic study with trypsin and chymotrypsin of peptide dendrimers from the dendrimer library (**D1**–**D9**) and their linear peptide analogues (**L1**–**L9**). The dendrimer sequences are given with the one letter code for proteinogenic amino acids. For the corresponding dendrimer structure see Figure 1.

Compound	Sequence	Yield[a] [mg]	MS calcd/found	Type[b]	Conversion[c]
D1	$(AcGFP)_4(BHypR^{\downarrow}M)_2BSG$-βAla-$NH_2$	23.2 (17%)	2665/2665	T	39%, 2 cuts
L1	$AcGFPAHypR^{\downarrow}MASG$-βAla-$NH_2$	49.8 (59%)	1118/1118		75%
D2	$(AcSFR^{\downarrow})_4(BVTP)_2BSG$-βAla-$NH_2$	24.3 (16%)	2814/2815	T	7.4%, 4 cuts
L2	$AcSFR^{\downarrow}AVTPASG$-βAla-$NH_2$	61.4 (73%)	1104/1104		58%
D3	$(AcGHA)_4(BVIM)_2BSG$-βAla-NH_2	19.2 (15%)	2406/2407	negT	0%
L3	$AcGHAAVIMASG$-βAla-NH_2	40.3 (51%)	1025/1025		0%
D4	$(AcDFP)_4(BHypRP)_2BSG$-βAla-NH_2	45.4 (32%)	2829/2829	negT	0%
L4	$AcDFPAHypRPASG$-βAla-NH_2	59.5 (69%)	1143/1142		0%
D5	$(AcGHR)_4(BVRM^{\downarrow})_2BSG$-βAla-$NH_2$	43.4 (21%)	2833/2833	Ch	39%
L5	$AcGHRAVRM^{\downarrow}ASG$-βAla-$NH_2$	35.0 (45%)	1154/1154		49%
D6	$(AcMF^{\downarrow}R)_4(BVRL)_2BSG$-βAla-$NH_2$	33.7 (17%)	3134/3134	Ch	100%
L6	$AcMF^{\downarrow}RAVRLASG$-βAla-$NH_2$	39.1 (52%)	1219/1219		100%
D7	$(AcGFP)_4(BHypTE)_2BSG$-βAla-NH_2	20.0 (15%)	2549/2550	negCh	0%
L7	$AcGFPAHypTEASG$-βAla-NH_2	33.7 (61%)	1062/1062		0%
D8	$(AcDER^{\downarrow})_4(BVIP)_2BSG$-βAla-$NH_2$	27.5 (18%)	2877/2877	desT	9%, 3 cuts
L8	$AcDER^{\downarrow}AVIPASG$-βAla-$NH_2$	27.5 (20%)	1127/1127		17%
D9	$(AcGEA)_4(BDR^{\downarrow}L)_2BSG$-βAla-$NH_2$	38.6 (31%)	2455/2457	desT	49%,[d] 1 or 2 cuts
L9	$AcGEAADR^{\downarrow}LASG$-βAla-$NH_2$	38.6 (33%)	1058/1058		15%[d]

[a] Yields were calculated from the weight of the TFA salts after RP-HPLC purification and lyophilization. *B*: (*S*)-2,3-diaminopropanoic acid or Dap, branching unit; Ac: acetyl; βAla: β-alanine; Hyp: 4-hydroxyproline. [b] Sequences from library **L** identified as trypsin reactive (T), trypsin unreactive (negT), α-chymotrypsin reactive (Ch), α-chymotrypsin unreactive (negCh), negative trypsin with an arginine mutation (desT). [c] The solution assays were run with dendrimer (1 mM) and trypsin (1 $\mu g\,mL^{-1}$) or α-chymotrypsin (50 $\mu g\,mL^{-1}$) in Tris buffer (0.1 M, pH 7.5). The reactions were followed by RP-HPLC at 214 nm and cleavage sites were determined by ESI-MS(+) analysis of the crude protease digests. [d] **D9** and **L9** did not cleave at 1 $\mu g\,mL^{-1}$ trypsin, the data shown here are for a trypsin concentration of 50 $\mu g\,mL^{-1}$.

Table 2. Synthesis of third generation fluorescein labeled peptide dendrimers and proteolysis results with trypsin.

Compound	Sequence	Yield [mg]	MS calcd/found	Type	Reactivity
D10	$(AcR^{\downarrow}A)_8BSD)_4(BYA)_2BALK$*-$NH_2$	0.7 (11.1%)	4750/4751	T	100%, 8 cuts
D11	$(AcHS)_8(BYF)_4(BR^{\downarrow}A)_2BFAK$*-$NH_2$	0.8 (13.8%)	5178/5179	T	100%, 2 cuts
D12	$(AcSA)_8(BAR^{\downarrow})_4(BAD)_2BFAK$*-$NH_2$	1.1 (9.8%)	4236/4237	T	100%, 4 cuts
D13	$(AcYA)_8(BR^{\downarrow}V)_4(BAD)_2BALK$*-$NH_2$	1.6 (43.8%)	4922/4924	T	100%, 4 cuts

Solution assays were performed with dendrimer (0.02 mM) and protease (50 $\mu g\,mL^{-1}$) in Tris buffer (0.1 M, pH 7.5). Reactions were followed by RP-HPLC at 440 nm and the cleavage sites were determined by using ESI-MS(+). Flow rate: 3.0 $mL\,min^{-1}$(A/D 70/30 to 50/50 in 10 min); K*: lysine with a FITC-labeled side chain; T: trypsin reactive.

resistant to proteolysis, specifically by using only one amino acid between branching points. To test this hypothesis, second generation (**D14**–**D16B/K**) and third generation (**D17**–**D19B/K**) dendrimers were prepared by combining a tryptic residue (Arg) and a chymotrypsin residue (Phe) with either small or polar residues (Gly, Ser) or an anionic residue (Glu). Each of the sequences was synthesized as a lysine-branched dendrimer, a more compact Dap-branched dendrimer, and a control linear peptide by using alanine at the branching position (Table 3).

Proteolysis assays with this series showed that the compact design was indeed able to protect protease reactive peptide sequences form proteolysis. The effect was most pronounced for the Dap branched dendrimers against trypsin. Thus, second generation dendrimers **D14B**–**D16B** underwent only 0–5% cleavage with trypsin, and third generation dendrimers **D17B**–**D19B** were completely resistant to trypsin and were also not cleaved significantly even after extended incubation times with up to 50 $\mu g\,mL^{-1}$ trypsin. By comparison the linear controls **L14**–**L19** were all readily cleaved. The lysine analogues **D14K**–**D19K** on the other hand were significantly cleaved, sometimes as much as their linear analogues (Table 3, Figure 3). Interestingly, analysis of the trypsin digests by MS showed that proteolysis occurred only once at the branching points with lysine, while the diaminopropanoic acid branching points sometimes also reacted twice. In the case of lysine hydrolysis at the ε-amino group the reaction is probably slower because the peptide bond is with a ε-amino rather than an α-amino group.

Proteolysis was also tested with α-chymotrypsin for sequences **14**, **18** and **19**, which contain a potential cleavage site for this protease. In this case the Dap-branched dendrimers **D14B**, **D18B** and **D19B** again showed reduced reactivity, with **D18B** being completely resistant to cleavage. On the other hand the lysine-branched dendrimers **D14K**, **D18K** and **D19K** and their linear analogues **L14**, **L18** and **L18** all underwent essentially complete cleavage.

The resistance of the Dap-branched dendrimers to proteolysis, which is particularly strong for the third generation dendrimers **D17B**–**D19B** compared to their lysine-branched analogues **D17K**–**D19K** or their linear analogues, might be caused by a compact structure reminiscent of a tightly folded protein. To investigate this point, the hydrodynamic radii of these third generation dendrimers were determined by diffusion NMR spectroscopy in D_2O (Table 4).[22] The data indicate that in each case the Dap-branched dendrimer was indeed significantly more compact than its lysine-branched analogue. However, the glutamate-containing dendrimers, **D18B/K** and **D19B/K**, were more resistant to proteases than **D17B/K** despite being less compact as measured by the hydrodynamic radius; this indicates that the

Table 3. Synthesis and proteolysis of compact peptide dendrimers incorporating one amino acid between diamino propionic acid as branching unit (**D14B**–**D19B**) or lysine as branching unit (**D14K**–**D19K**) and linear analogues (**L14**–**L19**). For the dendrimer structures see Figure 1.

Compound	Sequence	Yield[a] [mg]	MS calcd/found[b]	Trypsin	α-Chymotrypsin
D14B	$(AcF)_4(\mathit{B}R^{\downarrow})_2\mathit{B}L\text{-}NH_2$	27.9 (32%)	1458/1458	5.0%, 2 cuts	20%
D14K	$(AcF)_4(KR^{\downarrow})_2KL\text{-}NH_2$	34.4 (37%)	1584/1584	24%, 1 cut	96%
L14	$AcFAR^{\downarrow}AL\text{-}NH_2$	22.4 (59%)	618/618	90%	100%
D15B	$(AcE)_4(\mathit{B}R^{\downarrow})_2\mathit{B}L\text{-}NH_2$	24.8 (30%)	694[c]/693	0.4%, 1 cut	
D15K	$(AcE)_4(KR^{\downarrow})_2KL\text{-}NH_2$	22.9 (25%)	1510[c]/1511	40%, 1 cut	
L15	$AcEARAL\text{-}NH_2$	24.0 (65%)	600/600	19%	
D16B	$(AcG)_4(\mathit{B}R^{\downarrow})_2\mathit{B}L\text{-}NH_2$	27.2 (40%)	1097[c]/1098	4.5%, 1 or 2 cuts	
D16K	$(AcG)_4(KR^{\downarrow})_2KL\text{-}NH_2$	22.0 (29%)	1223/1223	46%, 1 cut	
L16	$AcGAR^{\downarrow}AL\text{-}NH_2$	25.0 (73%)	528/528	80%	
D17B	$(AcS)_8(\mathit{B}G)_4(\mathit{B}R^{\downarrow})_2\mathit{B}L\text{-}NH_2$	19.2 (12%)	2306/2306	0%,[e] 1 or 2 cuts	
D17K	$(AcS)_8(KG)_4(KR^{\downarrow})_2KL\text{-}NH_2$	13.5 (9%)	2601/2601	47%, 1 or 2 cuts	
L17	$AcSAGAR^{\downarrow}AL\text{-}NH_2$	25.7 (62%)	686/686	44%	
D18B	$(AcE)_8(\mathit{B}F)_4(\mathit{B}R^{\downarrow})_2\mathit{B}L\text{-}NH_2$	17.8 (9%)	3003/3003	0%[e]	0%
D18K	$(AcE)_8(KF)_4(KR^{\downarrow})_2KL\text{-}NH_2$	15.0 (8%)	3298/3298	18%, 1 cut	95%
L18	$AcEAFAR^{\downarrow}AL\text{-}NH_2$	31.6 (65%)	819/818	85%	100%
D19B	$(AcE)_8(\mathit{B}R^{\downarrow}{}_4(\mathit{B}F)_2\mathit{B}L\text{-}NH_2$	16.5 (7%)	3020/3021	0%[e]	26%
D19K	$(AcE)_8(KR^{\downarrow})_4(KF)_2KL\text{-}NH_2$	10.2 (5%)	3315/3316	2%[e] 2 cuts	100%
L19[d]	$AcEAR^{\downarrow}AFAL\text{-}NH_2$	23.7 (49%)	819/818	39%	94%

Conversion of dendrimers and linear peptides (1 mM) in Tris buffer (0.1 M, pH 7.5) after 1 h incubation with either trypsin (1 μg mL^{-1}) or α-chymotrypsin (50 μg mL^{-1}) at 25 °C. The digestions were analyzed by RP-HPLC at 214 nm after 1 h. [a] Yields were calculated for the TFA salts after RP-HPLC purification; [b] determined by ESI-MS(+); [c] $[M+2H]^{2+}$; [d] DMSO (20%) had to be added in order to solubilize this compound for the tests; [e] cleavage at 50 μg mL^{-1} trypsin cleaved **D18B** and **D19B** by less than 5%, **D17B** by 6% and **D19K** by 55%. *B*: (*S*)-2,3-diaminopropanoic acid branching unit; Ac: acetyl; βAla: β-alanine; Hyp: 4-hydroxyproline.

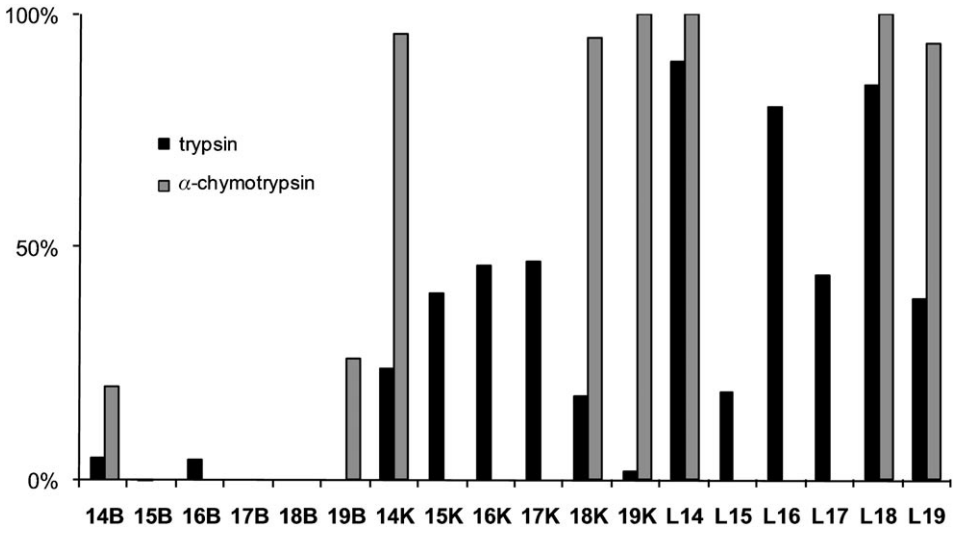

Figure 3. Reactivity of second and third generation peptide dendrimers and linear analogues by trypsin or chymotrypsin. The percentage of cleavage as detected by HPLC is given. Conditions: 1 mM dendrimer or peptide in Tris buffer (0.1 M, pH 7.5), trypsin (1 μg mL^{-1}) or α-chymotrypsin (50 μg mL^{-1}); samples were incubated for 1 h at 25 °C.

amino acid sequence also played a major role in reactivity. Interestingly, the volume per unit of molecular weight calculated from the hydrodynamic radius shows that dendrimers **D17B**/**K**–**D19B**/**K** (2.1–3.7 Å^3 mol g^{-1}) are as compact as folded proteins (about 2.5 Å^3 mol g^{-1}) and other peptide dendrimers, such as the esterase models[14] **RG1**–**RG3** and **RMG1**–**RMG3** (about 2.2–3.0 Å^3 mol g^{-1}; Table 4). By contrast denatured peptides and proteins occupy a much larger volume per unit of M_W (about 4–12 Å^3 mol g^{-1}). A general interpretation of the reduced protease reactivity in terms of compactness is therefore warranted, and most likely reflects reduced accessibility of the cleavage sites for the proteases.

Conclusions

The experiments described demonstrate that the protease reactivity of peptide dendrimers can be controlled by the frequency of branching diamino acids introduced in the sequence. Peptide dendrimers with three amino acids between branching points, such **D1** and analogues identified by a combinatorial on-bead proteolysis assay, undergo proteolysis at trypsin and α-chymotrypsin cleavage sites similar to their linear peptide analogues. The reactivity of these dendrimers primarily depends on the amino acid sequence, and unreactive sequences are readily identified, such as **D3**/**L3**, **D4**/**L4** and **D7**/**L7**. Reactivity is reduced in dendrimers with only two amino acids between branching points, such as dendrimers **D11** and **D12**. Protection of protease-reactive sequences from proteolysis by dendritic branching is finally achieved when only one amino acid is placed between branching points, in particular when using the short Dap residue as branching unit. Thus, the digestion sites within dendrimer **D18B** appear to be essentially untouched by either trypsin or α-chymotrypsin even after extended incubation times, while its linear peptide analogue **L18** is readily proteolyzed by both enzymes. This low reactivity is partly induced by the compact structure of the dendrimers, as evidenced by the estimation of their compactness by diffusion NMR spectroscopy.

While dendrimer branching and sequence design tune proteolysis, it should be mentioned that proteolysis in a peptide dendrimer should also be readily controllable by using unnatu-

Table 4. Hydrodynamic radii determined by diffusion NMR spectroscopy and specific volumes per M_W unit.

Compound	Sequence	M_W	r_H [nm]	V/M_W[c] [Å^3 mol g^{-1}]
D17B	$(AcS)_8(BG)_4(BR)_2BL\text{-}NH_2$	2306	1.06	2.14
D17K	$(AcS)_8(KG)_4(KR)_2KL\text{-}NH_2$	2601	1.16	2.48
D18B	$(AcE)_8(BF)_4(BR)_2BL\text{-}NH_2$	3003	1.22	2.52
D18K	$(AcE)_8(KF)_4(KR)_2KL\text{-}NH_2$	3298	1.43	3.69
D19B	$(AcE)_8(BR)_4(BF)_2BL\text{-}NH_2$	3020	1.24	2.66
D19K	$(AcE)_8(KR)_4(KF)_2KL\text{-}NH_2$	3315	1.28	2.63
RG1	$(AcRS)_2BHS\text{-}NH_2$	899	0.79	2.30
RG2	$(AcWG)_4(BRS)_2BHS\text{-}NH_2$	2127	1.04	2.19
RG3	$(AcYT)_8(BWG)_4(BRS)_2BHS\text{-}NH_2$	4752	1.44	2.64
RMG1	$(AcRSG)_2BHS\text{-}NH_2$	1013	0.86	2.63
RMG2	$(AcWG)_4(BRSG)_2BHS\text{-}NH_2$	2241	1.11	2.53
RMG3	$(AcYT)_8(BWG)_4(BRSG)_2BHS\text{-}NH_2$	4866	1.52	3.00
bovine pancreatic trypsin inhibitor[a]		6511	1.58	2.53
hen lysozyme[a]		14388	2.05	2.50
horse cytochrome c (NaCl-induced molten globule)[a]		12000	2.01	2.83
sperm whale apomyoglobin pH 4 (molten globule)[a]		17199	2.53	3.93
residues 2–38 from D3 of fibronectin binding protein[a,b]		3569	1.55	4.36
hen lysozyme[a,b]		14388	3.46	12.02

Diffusion NMR data were measured at a concentration of 1 mM of dendrimer/protein in D_2O at 303 K; r_H: hydrodynamic radius, *B*: (*S*)-2,3-diaminopropanoic acid. [a] Data from ref. [22]; [b] determined under strong denaturing conditions; [c] $V/M_W = (4/3)\times\pi\times r_H{}^3\times M_W{}^{-1}$.

ral amino acids, such as β-amino acids[23] or D-amino acids,[24] as is often done for linear peptides. For instance the prevalence of single cleavages of the dendrimers when the proteolytic site was placed directly at a branching point probably indicates the reduced reactivity of the side-chain amide group. In the perspective of biomedical applications, however, the use of unnatural building blocks should be taken with caution since they might induce unwanted toxicity. The control of proteolysis by topology provides a novel possibility to tune the biological properties of peptide dendrimers not available in linear peptides, and should be generally useful for their use as functional biomolecule analogues, in particular in the context of drug delivery applications.

Experimental Section

General: Reagents were purchased in the highest quality available from Fluka, Sigma, Bachem, Novabiochem, NeoMPS or Aldrich. All solvents used in reactions were bought in p.a. quality or distilled and dried prior to use. Solvents for extractions and chromatographic purifications were distilled from technical quality. Sensitive reactions were carried out under nitrogen or argon, and the glassware was heated under high vacuum. Chromatographic purifications (flash) were performed with silica gel 60 from Merck or Fluka (0.04 ± 0.063 nm; 230 ± 400 mesh, ASTM). TLC monitoring was performed with Alugram SIL G/UV254 silica gel sheets (Macherey–Nagel), followed by observation under a UV lamp (254/366 nm). Preparative RP-HPLC (flow rate 100 mL min^{-1}) was performed with a Waters Delta Prep 4000 system with a Waters Prepak Cartridge (500 g) as column and Waters 486 tunable absorbance detector. Semipreparative RP-HPLC (flow rate 4 mL min^{-1}) was performed with a Waters 510 pump operated with a Waters automated gradient controller and Jasco UV-2075 plus detector on a Vydac 218 TP (1.0 cm × 25 cm) column. Analytical RP-HPLC (flow rate 3 mL min^{-1}) was performed by using a Waters 600E systems with Waters Atlantis column (4.6 mm × 100 mm, dC18, 5 μm), and UV detection with Waters 996 photodiode array detector. Data recording and processing was done with Waters Empower2 software. Eluents for all systems were: A: water and TFA (0.1%); D: acetonitrile/water (3:2) with TFA (0.1%). MS analyses were provided by the mass spectrometry service of the Department of Chemistry and Biochemistry, University of Berne. The ^{1}H and ^{13}C NMR spectra were recorded by using a Bruker AC 300 (300 MHz) and DRX 500 or Avance 500 (500 MHz) instruments. Chemical shifts (δ) are given in ppm and refer to solvent residual peak, coupling constants (*J*) in Hertz (Hz). SPPS was performed manually in polypropylene syringes fitted with a polyethylene frit and a Teflon stopcock and stopper. Standard PGSE diffusion NMR spectroscopy experiments were performed by using a Bruker DRX500 in D_2O at 303 K. The gradient with a maximum strength of 50×10^{-4} T cm^{-1} was calibrated by using the HOD proton signal in 99.997% D_2O. The hydrodynamic radii were calculated from the diffusion coefficient D by using the Stokes–Einstein equation with $\eta = 1.095$ MPa s^{-1} for D_2O at 298 K.

Library synthesis

Coupling of Fmoc-protected amino acids: The on-bead library was synthesized on NovasynTG resin (Tentagel; 150 mg, 0.035 mmol, loading: 0.23 mmol g^{-1}) by using the split-and-mix procedure. The resin was swollen for 15 min in DCM, then it was acylated with 3 equiv of *N*-α-Fmoc amino acid in the presence of PyBOP (3 equiv, 58 mg, 0.1035 mmol) and *N,N'*-diisopropylethylamine ((*i*Pr)$_2$EtN; 6 equiv, 35 μL, 0.207 mmol) in DMF. After 2 × 60 min reaction times were prolonged for the amino acids (after one branching unit (60 min), after two branching units (90 min) and three branching units (120 min); for each branching unit twice the reaction time was used). The resin was washed (3 × 5 mL) with DMF, CH_2Cl_2 and MeOH and controlled with TNBS (trinitrobenzenesulfonic acid) test followed by acetylation.

Cleavage of the Fmoc protecting group: Fmoc protecting groups were removed by treatment with piperidine (20%) in DMF (3 mL, 3 × 10 min) and then washed with DMF, CH_2Cl_2 and MeOH.

Mix and split: Once the α-amine was deprotected, the resin was suspended in DMF/CH_2Cl_2 (2:1, *v/v*), mixed with nitrogen bubbling for 15 min and distributed in five equal portions for the next coupling.

N-Acetylation: The resin was acetylated with a solution of acetic acid anhydride/DCM (1:1, *v/v*) for 30 min. After filtration the resin was washed (3×) with DMF, MeOH and DCM.

TFA cleavage: The cleavage was carried out by using a TFA/H_2O/TIS/EDT (94:1:2.5:2.5, *v/v/v/v*) solution for 4 h.

On-bead proteolytic assays: Resin (50 mg) was equilibrated in THF/H_2O/AcOH (90:5:5, *v/v/v*) for 1 h. The solvent mixture was

then removed by filtration and 1 mL of a solution of formaldehyde (100 mM) in the same solvent mixture was added. After being stirred for 1 h, $NaCNBH_3$ (57 mg, 0.90 mmol) was added. The resin was stirred for 1 h and then washed with DMSO, DMF, MeOH, DCM, MeOH and finally with buffer (Tris buffer pH 7.5, 0.1 M). The resin was equilibrated in buffer for 2 h. After removal of the buffer by filtration, 1 mL of protease solution (1 mg mL^{-1}) was added and the mixture was stirred, overnight. The protease solution was removed by filtration and the resin was washed extensively with buffer, DMSO, DMF, MeOH, DCM, MeOH and finally with THF/H_2O/AcOH (90:5:5, *v/v/v*; 3×each). An aliquot of solution **1** (1 mL of 10 mM) in THF/H_2O/AcOH (90:5:5, *v/v/v*) was added and the resin was stirred for 1 h. $NaCNBH_3$ (6 mg, 0.09 mmol) was then added and the resin was stirred for 1 h. The labeling mixture was removed by filtration and the resin was washed extensively with DMSO, DMF, MeOH, DCM, MeOH and finally with H_2O. The resin was suspended in H_2O, transferred to a silica gel plate and the beads were observed under a microscope. Single red colored beads were transferred through a glass capillary to amino acid analysis vials. The following commercial enzymes were used: trypsin from pig pancreas (1645 U mg^{-1}; Fluka: #82495); and α-chymotrypsin from bovine pancreas (74.6 U mg^{-1}; Fluka: #27270).

Bead analysis: Single-dendrimer peptide-containing resin beads were hydrolyzed with aqueous HCl (6 M) at 110 °C for 22 h and the amino acid composition was determined quantitatively by HPLC after derivatization with phenyl isothiocyanate (PITC).

HPLC integration for sequence determination of single-peptide dendrimers on resin beads: The sequence of dendrimers was deduced from the HPLC-peak integration of each amino acid PITC derivative relative to the reference integration of this derivative. The Dap (2,3-diaminopropanoic acid) branching unit coelutes with phenylalanine. Thus, the value is subtracted before calculating integration for this amino acid.

Proteolytic stability test in solution: Peptides/dendrimers were prepared as 2 mM stock solutions in Tris buffer (0.1 M, pH 7.5). Proteolysis was initiated by the addition of 5 μL of a freshly prepared stock solution of protease (20 μg mL^{-1} trypsin, 1 mg mL^{-1} α-chymotrypsin) in Tris buffer (0.1 M, pH 7.5) to a mixture of peptide/dendrimer (50 μL) stock solution in Tris buffer (45 μL; 0.1 M, pH 7.5). The concentration under these conditions was 1 mM of peptide/dendrimer substrate and 1 μg mL^{-1} (trypsin) to 50 μg mL^{-1} (chymotrypsin) of protease. Reaction mixtures were analyzed after 1 h by RP-HPLC; (flow rate: 3.0 mL min^{-1}; gradient: A/D 100/0 to 0/100 in 15 min. Conversions were calculated by comparing the integrals of starting material after 1 h with a blank by using Empower2 software. If no new peaks were observed the conversion was assumed to be null. The crude proteolytic mixtures were subjected to MS (ESI+) analysis.

Solid-phase synthesis: Peptide dendrimers and linear peptides were synthesized by using NovasynTGR® (Tentagel with Rink amide linker) resin under the same coupling conditions as described for the peptide dendrimer library synthesis. After TFA cleavage the peptide dendrimers were separated from the resin by filtration and precipitated with MTBE. The crude product was dried under vacuum. It was then dissolved in a water/acetonitrile mixture and purified by preparative RP-HPLC.

Labeling of dendrimers with FITC: The dendrimer (2 μmol) was dissolved in K_2CO_3 solution (1 mL; 35 mM). FITC (fluorescein-5-isothiocyanate; 6 μmol) was dissolved in K_2CO_3 solution (1 mL; 35 mM) and was added to the dendrimer solution. The reaction mixture was stirred for 3 h at room temperature. The solution was then freeze-dried and the resulting solid was purified by semipreparative HPLC.

$(AcGlyPhePro)_4(DapHypArgMet)_2DapSerGlyBla\text{-}NH_2$ (D1): From NovasynTGR (200 mg, 0.23 mmol g^{-1}), **D1** was obtained as a foamy colorless solid after preparative RP-HPLC (23.2 mg, 7.82 μmol, 17%). RP-HPLC: t_R = 13.0 min (A/D 100/0 to 0/100 in 15 min, λ = 214 nm); ESI MS(+): calcd for $C_{121}H_{174}N_{34}O_{31}S_2$ *m/z*: 2665 $[M+H]^+$; found: 2665 $[M+H]^+$.

$AcGlyPheProAlaHypArgMetAlaSerGlyBla\text{-}NH_2$ (L1): From NovasynTGR (300 mg, 0.23 mmol g^{-1}), **L1** was obtained as a foamy colorless solid after preparative RP-HPLC (49.8 mg, 40.7 μmol, 59%). RP-HPLC: t_R = 6.9 min (A/D 100/0 to 0/100 in 15 min, λ = 214 nm); ESI MS(+): calcd for $C_{48}H_{75}N_{15}O_{14}S$ *m/z*: 1118 $[M+H]^+$; found: 1118.

$(AcSerPheArg)_4(DapValThrPro)_2DapSerGlyBla\text{-}NH_2$ (D2): From NovasynTGR (200 mg, 0.23 mmol g^{-1}), **D2** was obtained as a foamy colorless solid after preparative RP-HPLC (24.3 mg, 7.36 μmol, 16%). RP-HPLC: t_R = 8.7 min (A/D 100/0 to 0/100 in 15 min, λ = 214 nm); ESI MS(+): calcd for $C_{125}H_{192}N_{40}O_{35}$ *m/z*: 2815 $[M+H]^+$; found: 2815 $[M+H]^+$.

$AcSerPheArgAlaValThrProAlaSerGlyBla\text{-}NH_2$ (L2): From NovasynTGR (300 mg, 0.23 mmol g^{-1}), **L2** was obtained as a foamy colorless solid after preparative RP-HPLC (61.4 mg, 50.4 μmol, 73%). RP-HPLC: t_R = 7.1 min (A/D 100/0 to 0/100 in 15 min, λ = 214 nm); ESI MS(+): calcd for $C_{48}H_{77}N_{15}O_{15}$ *m/z*: 1104 $[M+H]^+$; found: 1104 $[M+H]^+$.

$(AcGlyHisAla)_4(DapValIleMet)_2DapSerGlyBla\text{-}NH_2$ (D3): From NovasynTGR (200 mg, 0.23 mmol g^{-1}), **D3** was obtained as a foamy colorless solid after preparative RP-HPLC (19.2 mg, 6.90 μmol, 15%). RP-HPLC: t_R = 7.7 min (A/D 100/0 to 0/100 in 15 min, λ = 214 nm); ESI MS(+): calcd for $C_{101}H_{164}N_{36}O_{29}S_2$ *m/z*: 2407 $[M+H]^+$; found: 2407 $[M+H]^+$.

$AcGlyHisAlaAlaValIleMetAlaSerGlyBla\text{-}NH_2$ (L3): From NovasynTGR (300 mg, 0.23 mmol g^{-1}), **L3** was obtained as a foamy colorless solid after preparative RP-HPLC (40.3 mg, 35.2 μmol, 51%). RP-HPLC: t_R = 6.9 min (A/D 80/60 to 60/40 in 15 min, λ = 214 nm); ESI MS(+): calcd for $C_{43}H_{72}N_{14}O_{13}S$ *m/z*: 1025 $[M+H]^+$; found: 1025 $[M+H]^+$.

$(AcAspPhePro)_4(DapHypArgPro)_2DapSerGlyBla\text{-}NH_2$ (D4): From NovasynTGR (200 mg, 0.23 mmol g^{-1}), **D4** was obtained as a foamy colorless solid after preparative RP-HPLC (45.4 mg, 14.7 μmol, 32%). RP-HPLC: t_R = 9.2 min (A/D 100/0 to 0/100 in 15 min, λ = 214 nm); ESI MS(+): calcd for $C_{129}H_{178}N_{34}O_{39}$ *m/z*: 2829 $[M+H]^+$; found: 2829 $[M+H]^+$.

$AcAspPheProAlaHypArgProAlaSerGlyBla\text{-}NH_2$ (L4): From NovasynTGR (300 mg, 0.23 mmol g^{-1}), **L4** was obtained as a foamy colorless solid after preparative RP-HPLC (59.5 mg, 47.6 μmol, 69%). RP-HPLC: t_R = 6.4 min (A/D 100/0 to 0/100 in 15 min, λ = 214 nm); ESI MS(+): calcd for $C_{50}H_{75}N_{15}O_{16}$ *m/z*: 1143 $[M+H]^+$; found: 1143 $[M+H]^+$.

$(AcGlyHisArg)_4(DapValArgMet)_2DapSerGlyBla\text{-}NH_2$ (D5): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **D5** was obtained as a foamy colorless solid after preparative RP-HPLC (43.4 mg, 10.9 μmol, 21%). RP-HPLC: t_R = 6.7 min (A/D 100/0 to 0/100 in 15 min, λ = 214 nm); ESI MS(+): calcd for $C_{113}H_{190}N_{54}O_{29}S_2$ *m/z*: 2833 $[M+H]^+$; found: 2833 $[M+H]^+$.

$AcGlyHisArgAlaValArgMetAlaSerGlyBla\text{-}NH_2$ (L5): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **L5** was obtained as a foamy colorless solid after preparative RP-HPLC (35.0 mg, 23.4 μmol, 45%).

RP-HPLC: $t_R = 5.9$ min (A/D 100/0 to 0/100 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{46}H_{80}N_{20}O_{13}S$ *m/z*: 1154 $[M+H]^+$; found: 1268 $[M+TFA+H]^+$.

(AcMetPheArg)$_4$(DapValArgLeu)$_2$DapSerGlyBla-NH$_2$ (D6): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **D6** was obtained as a foamy colorless solid after preparative RP-HPLC (33.7 mg, 8.84 μmol, 17%). RP-HPLC: $t_R = 11.6$ min (A/D 100/0 to 0/100 in 15 min, $\lambda =$ 214 nm); ESI MS(+): calcd for $C_{139}H_{226}N_{46}O_{29}S_4$ *m/z*: 3134 $[M+H]^+$; found: 3134 $[M+H]^+$.

AcMetPheArgAlaValArgLeuAlaSerGlyBla-NH$_2$ (L6): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **L6** was obtained as a foamy colorless solid after preparative RP-HPLC (39.1 mg, 27.0 μmol, 52%). RP-HPLC: $t_R = 9.6$ min (A/D 100/0 to 0/100 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{53}H_{90}N_{18}O_{13}S$ *m/z*: 1219 $[M+H]^+$; found: 1219 $[M+H]^+$.

(AcGlyPhePro)$_4$(DapHypThrGlu)$_2$DapSerGlyBla-NH$_2$ (D7): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **D7** was obtained as a foamy colorless solid after preparative RP-HPLC (20.0 mg, 7.80 μmol, 15%). RP-HPLC: $t_R = 10.1$ min (A/D 80/20 to 60/40 in 15 min, $\lambda =$ 214 nm); ESI MS(+): calcd for $C_{117}H_{160}N_{28}O_{37}$ *m/z*: 2549 $[M+H]^+$; found: 2550 $[M+H]^+$.

AcGlyPheProAlaHypThrGluAlaSerGlyBla-NH$_2$ (L7): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **L7** was obtained as a foamy colorless solid after preparative RP-HPLC (33.7 mg, 31.7 μmol, 61%). RP-HPLC: $t_R = 6.7$ min (A/D 100/0 to 0/100 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{46}H_{68}N_{12}O_{17}$ *m/z*: 1062 $[M+H]^+$; found: 1062 $[M+H]^+$.

(AspGluArg)$_4$(DapValIlePro)$_2$DapSerGlyBla-NH$_2$ (D8): From NovasynTGR (200 mg, 0.23 mmol g^{-1}), **D8** was obtained as a foamy colorless solid after preparative RP-HPLC (27.5 mg, 8.25 μmol, 18%). RP-HPLC: $t_R = 10.8$ min (A/D 80/20 to 60/40 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{117}H_{192}N_{40}O_{45}$ *m/z*: 2877 $[M+H]^+$; found: 2877 $[M+H]^+$.

AspGluArgAlaValIleProAlaSerGlyBla-NH$_2$ (L8): From NovasynTGR (200 mg, 0.23 mmol g^{-1}), **L8** was obtained as a foamy colorless solid after preparative RP-HPLC (27.5 mg, 9.19 μmol, 20%). RP-HPLC: $t_R = 6.9$ min (A/D 100/0 to 0/100 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{47}H_{79}N_{15}O_{17}$ *m/z*: 1127 $[M+H]^+$; found: 1127 $[M+H]^+$.

(AcGlyGluAla)$_4$(DapAspArgLeu)$_2$DapSerGlyBla-NH$_2$ (D9): From NovasynTGR (200 mg, 0.23 mmol g^{-1}), **D9** was obtained as a foamy colorless solid after preparative RP-HPLC (38.6 mg, 14.38 μmol, 31%). RP-HPLC: $t_R = 7.6$ min (A/D 100/0 to 0/100 in 15 min, $\lambda =$ 214 nm); ESI MS(+): calcd for $C_{97}H_{158}N_{34}O_{41}$ *m/z*: 2455 $[M+H]^+$; found: 2457 $[M+H]^+$.

AcGlyGluAlaAlaAspArgLeuAlaSerGlyBla-NH$_2$ (L9): From NovasynTGR (200 mg, 0.23 mmol g^{-1}), **L9** was obtained as a foamy colorless solid after preparative RP-HPLC (38.6 mg, 15.0 μmol, 33%). RP-HPLC: $t_R = 6.8$ min (A/D 100/0 to 0/100 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{42}H_{71}N_{15}O_{17}$ *m/z*: 1058 $[M+H]^+$; found: 1058.

(AcArgAla)$_8$(DapSerAsp)$_4$(DapTyrAla)$_2$DapAlaLeuLys(FITC)-NH$_2$ (D10): From NovasynTGR (100 mg, 0.25 mmol g^{-1}), the peptide dendrimer (AcRA)$_8$(DapSD)$_4$(DapYA)$_2$DapALK-NH$_2$ was obtained as a foamy colorless solid after preparative RP-HPLC (5.9 mg, 1.35 μmol, 3.6%). RP-HPLC: $t_R = 4.2$ min (A/D 80/20 to 60/40 in 10 min, $\lambda =$ 214 nm); MS (ESI+): $C_{176}H_{293}N_{71}O_{60}$ calcd: 4361; found: 4363 $[M]^+$. The dendrimer was then conjugated with FITC as described above to yield **D10** as a yellow solid after semipreparative RP-HPLC (0.7 mg, 0.15 μmol, 11.1%). RP-HPLC: $t_R = 5.6$ min (A/D 70/30 to 50/50 in 10 min, $\lambda = 214$ nm); MS (ESI+): $C_{197}H_{304}N_{72}O_{65}S$ calcd: 4750; found: 4751 $[M]^+$.

(AcHisSer)$_8$(DapTyrPhe)$_4$(DapArgAla)$_2$DapPheAlaLys(FITC)-NH$_2$ (D11): From NovasynTGR (100 mg, 0.25 mmol g^{-1}) the peptide dendrimer (AcHS)$_8$(DapYF)$_4$(DapRA)$_2$DapFAK-NH$_2$ was obtained as a foamy colorless solid after preparative RP-HPLC (5.2 mg, 1.09 μmol, 4.4%). RP-HPLC: $t_R = 7.1$ min (A/D 75/25 to 55/45 in 10 min, $\lambda =$ 214 nm); MS (ESI+): $C_{217}H_{289}N_{69}O_{58}$ calcd: 4789; found: 4791 $[M]^+$. The dendrimer was then conjugated with FITC as described above to yield **D11** as a yellow solid after semipreparative RP-HPLC (0.8 mg, 0.15 μmol, 13.8%). RP-HPLC: $t_R = 6.7$ min (A/D 70/30 to 50/50 in 10 min, $\lambda = 214$ nm); MS (ESI+): $C_{238}H_{300}N_{70}O_{63}S$ calcd: 5179; found: 5178 $[M]^+$.

(AcSerAla)$_8$(DapAlaArg)$_4$(DapAlaAsp)$_2$DapAlaLeuLys(FITC)-NH$_2$ (D12): From NovasynTGR (100 mg, 0.25 mmol g^{-1}) the peptide dendrimer (AcSA)$_8$(DapAR)$_4$(DapAD)$_2$DapALK-NH$_2$ was obtained as a foamy colorless solid after preparative RP-HPLC (10.2 mg, 2.65 μmol, 10.6%). RP-HPLC: $t_R = 5.7$ min (A/D 85/15 to 65/35 in 10 min, $\lambda = 214$ nm); MS (ESI+): $C_{153}H_{255}N_{59}O_{58}$ calcd: 3847; found: 3848 $[M]^+$. The dendrimer was then conjugated with FITC as described above to yield **D12** as a yellow solid after semipreparative RP-HPLC (1.1 mg, 0.26 μmol, 9.8%). RP-HPLC: $t_R = 4.6$ min (A/D 70/30 to 50/50 in 10 min, $\lambda = 214$ nm); MS (ESI+): $C_{174}H_{266}N_{60}O_{63}S$ calcd: 4236; found: 4238 $[M]^+$.

(AcTyrAla)$_8$(DapArgVal)$_4$(DapAlaAsp)$_2$DapAlaLeuLys(FITC)-NH$_2$ (D13): From NovasynTGR (100 mg, 0.25 mmol g^{-1}), the peptide dendrimer (AcYA)$_8$(DapRV)$_4$(DapAD)$_2$DapALK-NH$_2$ was obtained as a foamy colorless solid after preparative RP-HPLC (3.3 mg, 0.73 μmol, 2.9%). RP-HPLC: $t_R = 6.7$ min (A/D 70/30 to 50/50 in 10 min, $\lambda =$ 214 nm); MS (ESI+): $C_{206}H_{305}N_{59}O_{58}$ calcd: 4533; found: 4534 $[M]^+$. The dendrimer was then conjugated with FITC as described above to yield **D13** as a yellow solid after semipreparative RP-HPLC (1.6 mg, 0.32 μmol, 43.8%). RP-HPLC: $t_R = 4.8$ min (A/D 60/40 to 40/60 in 10 min, $\lambda = 214$ nm); MS (ESI+): $C_{227}H_{316}N_{60}O_{63}S$ calcd: 4922; found: 4924 $[M]^+$.

(AcPhe)$_4$(DapArg)$_2$DapLeu-NH$_2$ (D14B): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **D14B** was obtained as a foamy colorless solid after preparative RP-HPLC (27.9 mg, 16.6 μmol, 32%). RP-HPLC: $t_R = 12.1$ min (A/D 100/0 to 0/100 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{71}H_{100}N_{20}O_{14}$ *m/z*: 1458 $[M+H]^+$; found: 1458.

(AcPhe)$_4$(LysArg)$_2$LysLeu-NH$_2$ (D14K): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **D14K** was obtained as a foamy colorless solid after preparative RP-HPLC (34.4 mg, 19.0 μmol, 37%). RP-HPLC: $t_R =$ 12.4 min (A/D 100/0 to 0/100 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{80}H_{118}N_{20}O_{14}$ *m/z*: 1584 $[M+H]^+$; found: 1584.

AcPheAlaArgAlaLeu-NH$_2$ (L14): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **L14** was obtained as a foamy colorless solid after preparative RP-HPLC (22.4 mg, 30.6 μmol, 59%). RP-HPLC: $t_R =$ 8.9 min (A/D 100/0 to 0/100 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{29}H_{47}N_9O_6$ *m/z*: 618 $[M+H]^+$; found: 618 $[M+H]^+$.

(AcGlu)$_4$(DapArg)$_2$DapLeu-NH$_2$ (D15B): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **D15B** was obtained as a foamy colorless solid after preparative RP-HPLC (24.8 mg, 15.4 μmol, 30%). RP-HPLC: $t_R = 6.5$ min (A/D 100/0 to 0/100 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{55}H_{92}N_{20}O_{22}$ *m/z*: 1384 $[M+H]^+$; found: 1385 $[M+H]^+$.

(AcGlu)$_4$(LysArg)$_2$LysLeu-NH$_2$ (D15K): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **D15K** was obtained as a foamy colorless solid after preparative RP-HPLC (22.9 mg, 13.2 μmol, 25%). RP-HPLC: $t_R =$

7.1 min (A/D 100/0 to 0/100 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{64}H_{110}N_{20}O_{22}$ *m/z*: 1510 $[M+H]^+$; found: 1511 $[M+H]^+$.

AcGluAlaArgAlaLeu-NH$_2$ (L15): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **L15** was obtained as a foamy colorless solid after preparative RP-HPLC (24.0 mg, 33.6 μmol, 65%). RP-HPLC: $t_R =$ 6.2 min (A/D 100/0 to 0/100 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{25}H_{45}N_9O_8$ *m/z*: 600 $[M+H]^+$; found: 600 $[M+H]^+$.

(AcGly)$_4$(DapArg)$_2$DapLeu-NH$_2$ (D16B): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **D16B** was obtained as a foamy colorless solid after preparative RP-HPLC (27.4 mg, 20.7 μmol, 40%). RP-HPLC: $t_R = 6.0$ min (A/D 100/0 to 0/100 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{43}H_{76}N_{20}O_{14}$ *m/z*: 1097 $[M+H]^+$; found: 1098 $[M+H]^+$.

(AcGly)$_4$(LysArg)$_2$LysLeu-NH$_2$ (D16K): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **D16K** was obtained as a foamy colorless solid after preparative RP-HPLC (22.0 mg, 15.2 μmol, 29%). RP-HPLC: $t_R =$ 6.7 min (A/D 100/0 to 0/100 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{52}H_{94}N_{20}O_{14}$ *m/z*: 1223 $[M+H]^+$; found: 1223 $[M+H]^+$.

AcGlyAlaArgAlaLeu-NH$_2$ (L16): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **L16** was obtained as a foamy colorless solid after preparative RP-HPLC (25.0 mg, 38.9 μmol, 73%). RP-HPLC: $t_R =$ 6.0 min (A/D 100/0 to 0/100 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{22}H_{41}N_9O_6$ *m/z*: 528 $[M+H]^+$; found: 528 $[M+H]^+$.

(AcSer)$_8$(DapGly)$_4$(DapArg)$_2$DapLeu-NH$_2$ (D17B): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **D17B** was obtained as a foamy colorless solid after preparative RP-HPLC (19.2 mg, 7.57 μmol, 12%). RP-HPLC: $t_R = 6.2$ min (A/D 100/0 to 0/100 in 10 min, $\lambda =$ 214 nm); ESI MS(+): calcd for $C_{87}H_{148}N_{36}O_{38}$ *m/z*: 2306 $[M+H]^+$; found: 2306 $[M+H]^+$.

(AcSer)$_8$(LysGly)$_4$(LysArg)$_2$LysLeu-NH$_2$ (D17K): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **D17K** was obtained as a foamy colorless solid after preparative RP-HPLC (13.5 mg, 4.77 μmol, 9%). RP-HPLC: $t_R = 6.8$ min (A/D 100/0 to 0/100 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{108}H_{150}N_{36}O_{38}$ *m/z*: 2601 $[M+H]^+$; found: 2601 $[M+H]^+$.

AcSerAlaGlyAlaArgAlaLeu-NH$_2$ (L17): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **L17** was obtained as a foamy colorless solid after preparative RP-HPLC (25.7 mg, 32.1 μmol, 62%). RP-HPLC: $t_R =$ 6.3 min (A/D 100/0 to 0/100 in 10 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{28}H_{51}N_{11}O_9$ *m/z*: 686 $[M+H]^+$; found: 686 $[M+H]^+$.

(AcGlu)$_8$(DapPhe)$_4$(DapArg)$_2$DapLeu-NH$_2$ (D18B): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **D18B** was obtained as a foamy colorless solid after preparative RP-HPLC (17.8 mg, 5.50 μmol, 9%). RP-HPLC: $t_R = 10.1$ min (A/D 100/0 to 0/100 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{131}H_{188}N_{36}O_{46}$ *m/z*: 3003 $[M+H]^+$; found: 3003 $[M+H]^+$.

(AcGlu)$_8$(LysPhe)$_4$(LysArg)$_2$LysLeu-NH$_2$ (D18K): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **D18K** was obtained as a foamy colorless solid after preparative RP-HPLC (15.0 mg, 4.26 μmol, 8%). RP-HPLC: $t_R = 10.8$ min (A/D 100/0 to 0/100 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{152}H_{230}N_{36}O_{46}$ *m/z*: 3298 $[M+H]^+$; found: 3298 $[M+H]^+$.

AcGluAlaPheAlaArgAlaLeu-NH$_2$ (L18): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **L18** was obtained as a foamy colorless solid after preparative RP-HPLC (31.6 mg, 33.9 μmol, 65%). RP-HPLC: $t_R =$ 9.7 min (A/D 100/0 to 0/100 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{37}H_{59}N_{11}O_{10}$ *m/z*: 819 $[M+H]^+$; found: 818 $[M+H]^+$.

(AcGlu)$_8$(DapArg)$_4$(DapPhe)$_2$DapLeu-NH$_2$ (D19B): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **D19B** was obtained as a foamy colorless solid after preparative RP-HPLC (16.5 mg, 4.75 μmol, 7%). RP-HPLC: $t_R = 8.4$ min (A/D 100/0 to 0/100 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{125}H_{194}N_{42}O_{46}$ *m/z*: 3021 $[M+H]^+$; found: 3021 $[M+H]^+$.

(AcGlu)$_8$(LysArg)$_4$(LysPhe)$_2$LysLeu-NH$_2$ (D19K): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **D19K** was obtained as a foamy colorless solid after preparative RP-HPLC (10.2 mg, 2.70 μmol, 5%). RP-HPLC: $t_R = 9.1$ min (A/D 100/0 to 0/100 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{146}H_{236}N_{42}O_{46}$ *m/z*: 3315 $[M+H]^+$; found: 3315 $[M+H]^+$.

AcGluAlaArgAlaPheAlaLeu-NH$_2$ (L19): From NovasynTGR (200 mg, 0.26 mmol g^{-1}), **L19** was obtained as a foamy colorless solid after preparative RP-HPLC (23.7 mg, 25.4 μmol, 49%). RP-HPLC: $t_R =$ 8.9 min (A/D 100/0 to 0/100 in 15 min, $\lambda = 214$ nm); ESI MS(+): calcd for $C_{37}H_{59}N_{11}O_{10}$ *m/z*: 819 $[M+H]^+$; found: 818 $[M+H]^+$.

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