Supplementary Informations for:

Inhibition of *Pseudomonas aeruginosa* biofilms with a glycopeptide dendrimer containing D-amino acids

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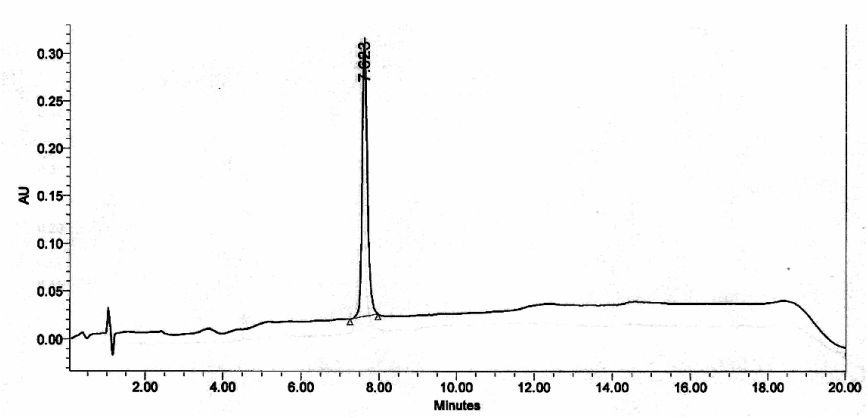
Synthesis

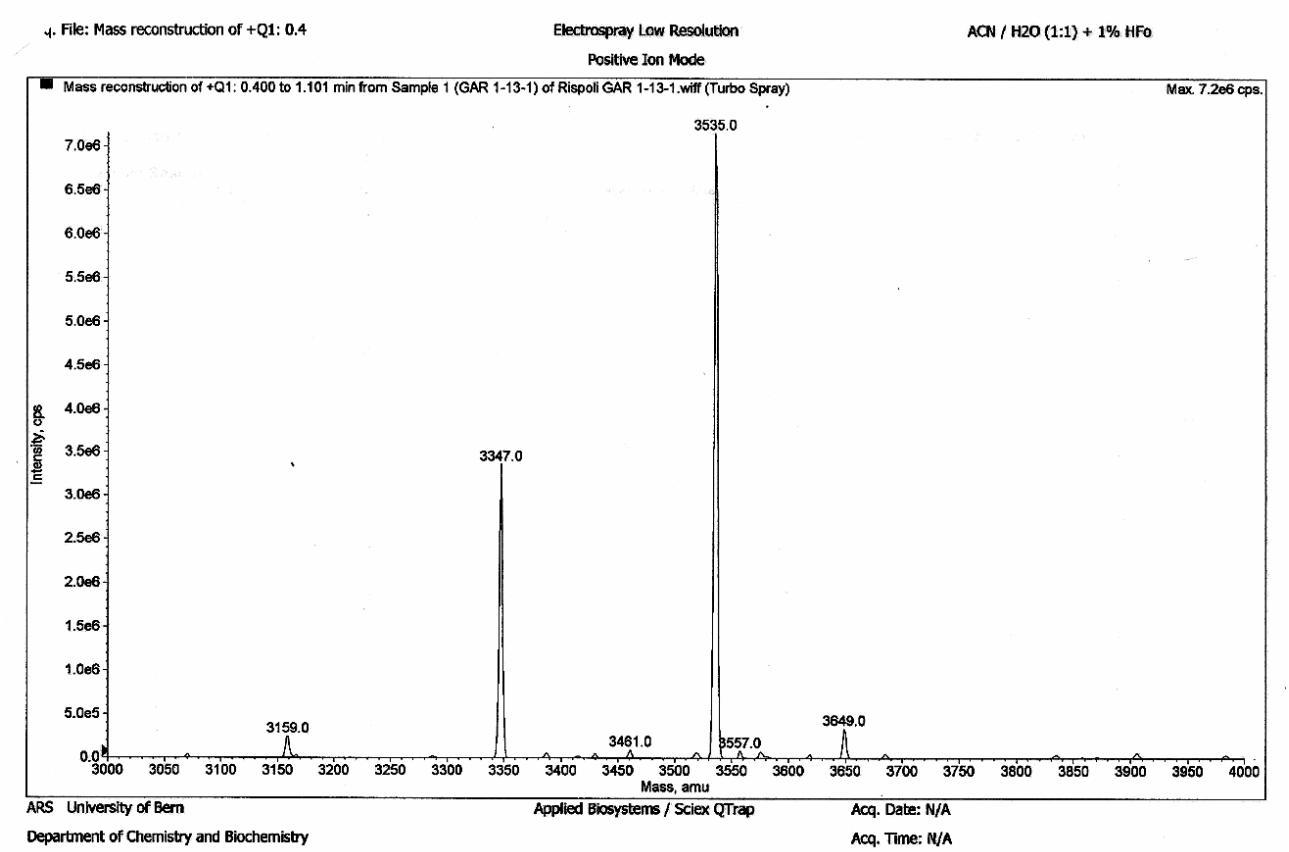
Materials and reagents. The L- and D-amino acids were used as the following derivatives: Fmoc-His(Boc)-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Ile-OH, Fmoc-Pro-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(Fmoc)-OH, Rink amide NovaSyn TGR resin were purchased from Novabiochem (Switzerland). Chemicals were used as supplied and solvents were of analytical grade, human serum from Aldrich (Switzerland). Analytical RP-HPLC was performed in Waters (996 Photo diode array detector) chromatography system using a chromolith performance RP-C18, 4.6 x 100 mm, flow rate 3 $mL.min^{-1}$ column. Compounds were detected by UV absorption at 214 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 or 1.25 $\%.min^{-1}$ CH_3CN). MS spectra were provided by the Service of Mass Spectrometry of the Department of Chemistry and Biochemistry, University of Bern.

Procedure for dendrimer synthesis. For synthesis and characterization of dendrimers **PA5** and **PA6** and characterization of **FD2** see reference [1]

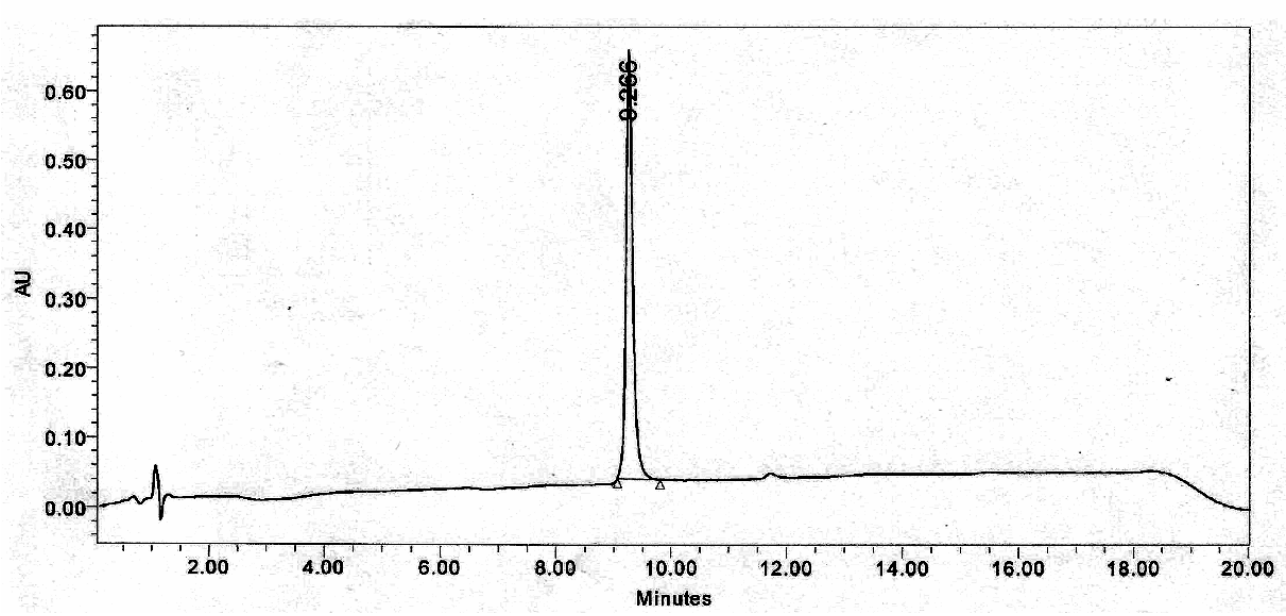
Synthesis of FD2, D**-FD2 and Leu-FD2.** Peptide syntheses were performed manually in a glass reactor or plastic syringes (5 or 10 mL). The resin NovaSyn® TGR (loading: 0.18-0.29 mmol/g) was acylated with each amino acid or diamino acid (3 eq) in the presence of BOP or PyBOP (3 eq) and DIEA (5 eq) for 1 h and 30 min, 3 h after the first generation. After each coupling the resin was successively washed with NMP, MeOH, and CH_2Cl_2 (3× with each solvent), then checked for free amino groups with the TNBS test. If the TNBS test indicated the presence of free amino groups, the coupling was repeated. After each coupling the potential remaining free amino groups were capped with acetic anhydride/CH_2Cl_2 for 10 min. The Fmoc protecting groups were removed with a solution of 20% piperidine in DMF (2×10 min) and the solvent was removed by filtration. In the end of the sequence the resin was capped with 2-(Tri-O-acetyl-α-L-fucopyranosyl)acetic acid (5 eq) in the presence of DIC (5 eq) and HOBt (5 eq) or DIEA (5 eq) and HCTU (3 eq) in NMP overnight. The carbohydrate was deprotected with a solution of MeOH/NH_4/H_2O (v/v 8:1:1) for 24 h. The resin was dried and the cleavage was carried out with TFA/TIS/H_2O (95:2.5:2.5) for 4 h. The peptide was precipitated with methyl *tert*-butyl ether then dissolved in a water/acetonitrile mixture. All dendrimers were purified by preparative HPLC with detection at $\lambda = 214$ nm. Eluent A: water and TFA (0.1%); eluent B: acetonitrile, water and TFA (3/2/0.1%).

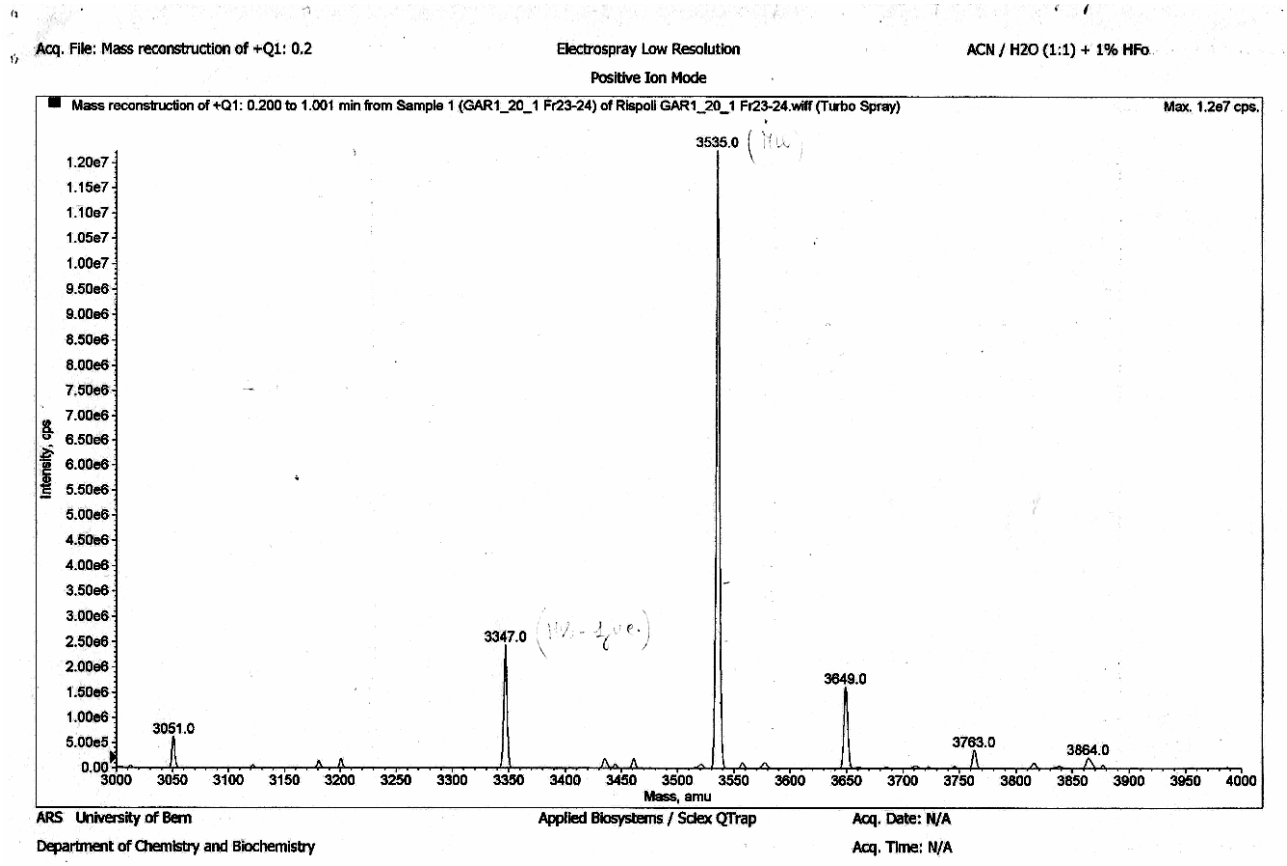
D-FD2: ((CFuc-lys-pro-leu)$_2$*Lys*-phe-lys-leu)$_2$*Lys*-his-leuNH$_2$ (from 300 mg resin 0.24 mmol/g, 30.0 mg, 12 %). MS (ES+) calc for $C_{172}H_{290}N_{35}O_{43}$ $[M+H]^+$: 3536.34, found: 3535. Preparative RP-HPLC: (A/B = 80/20 to A/B = 40/60 in 50 min).





Leu-FD2: ((CFuc-Lys-Pro-Leu)$_2$*Lys*-Phe-Lys-Leu)$_2$*Lys*-His-LeuNH$_2$. (from 300 mg resin 0.24 mmol/g, 16.2 mg, 13.9 %). MS (ES+) calc for $C_{172}H_{290}N_{35}O_{43}$ $[M+H]^+$: 3536.34, found: 3535.0. Preparative RP-HPLC: (A/B = 80/20 to A/B = 40/60 in 50 min).





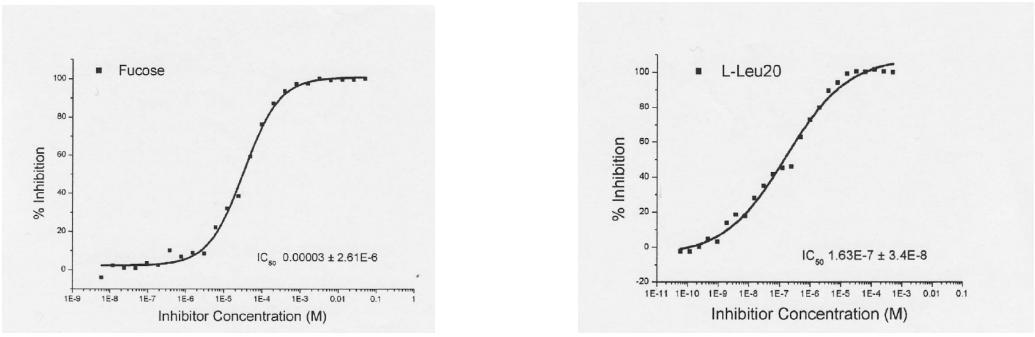
Expression and purification of LecB The procedure for lectin expression and purification has been described[1] The *lecB* gene of *P. aeruginosa* on plasmid pEC2 was expressed in *Escherichia coli* BL21(DE3). Bacterial cells were grown at 37°C in Luria-Bertani medium containing 0.4% (w/v) glucose to an absorbance at 580 nm of 0.6 and then induced with 0.5 mM isopropyl-β-D-thiogalactoside. After additional 16h of growth, cells were harvested by centrifugation at 8000 x g for 10 min and suspended in 20 ml of 100 mM Tris.HCl buffer, pH 8.0. LecB was purified by affinity chromatography after lysis of the bacterial cells by sonication.. The LecB yield usually was 60 mg per liter of culture, the pure lectin protein was stored in aliquots at a concentration of 1mg/ml at -20°C.

Assays

ELLA (enzyme-linked lectin assays)

All ELLA assays were done at the same time for dendrimers and controls, with the same batches of LecB and reagents. ELLAs were conducted using 96-well microtitre plates (Nunc Maxisorb) coated with LecB lectin (5 µg/mL) diluted in carbonate buffer, pH 9.6 (100 µL) for 1 h at 37 °C. After removal of lectin, the wells were blocked with 100 µL per well of 3% (w/v) BSA in PBS at 37 °C for 1 h. BSA solution was removed and each inhibitor was added in serial 2-fold dilutions (54 µL/well) in PBS to lectin-coated microplates and incubated at 37 °C for 1 h. Then 54 µL of biotinylated polymeric fucose (Lectinity Holding, Inc.) at 5 µg/mL was added to the above solutions of inhibitors and the plates were incubated for another hour at 37 °C. After washing (3× 150 µL/well with T-PBS (PBS containing 0.05% Tween), 100 µL of streptavidin–peroxidase conjugate (dilution 1:5000 in PBS) was added and left for 1 h at 37 °C. The wells were then washed three times with 150 µL/well with T-PBS and once with water, and 50 µL/well of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (1 mg per 4 mL) in citrate phosphate buffer (0.2 M, pH 4.0 with 0.015 % (v/v) H_2O_2) was added. The reaction was stopped after 20 min by adding 50 µL/well of 1 M H_2SO_4. Absorbance was read at 415 nm using a microtiter plate reader (spectra MAX 250). Every experiment was made in triplicate.

a) Fucose **b) Leu-FD2**



c) FD2 **d) D-FD2**

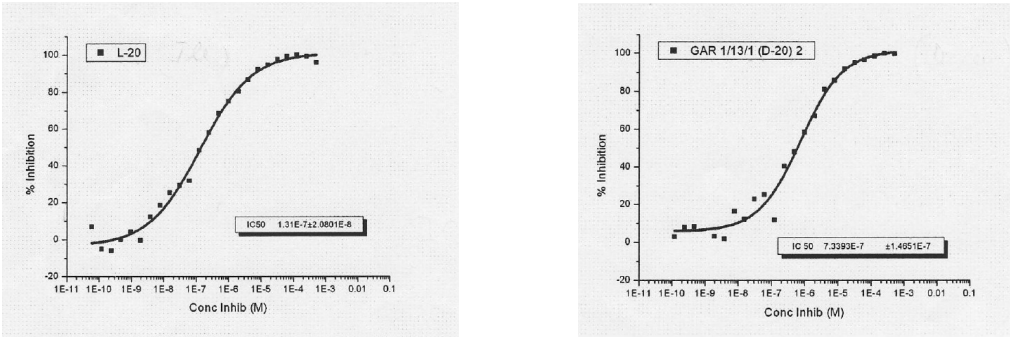


Figure S1. Representative ELLA plots for dendrimers and reference compound with *P. aeruginosa* lectin LecB performed in this study.

Circular dichroism (CD)

The CD Spectra was recorded using a Jasco J-715 spectropolarimeter equipped with a PFD-350S temperature controller and a PS-150 J power supply. All the experiments were performed using Hellma Suprasil® 110-QS 0.1 cm cuvettes. Freshly prepared stock solutions (1.0 mg×mL^{-1} in Milli-Q water) of the peptide dendrimers as TFA salt were used for the measurements. The scan rate was 10 nm×min^{-1}, pitch 0.5 nm, response 16 sec and band 1.0 nm. Single scans were performed and the nitrogen flow was kept at 9 L×min^{-1}. The sample concentrations were 0.10 mg×mL^{-1} and 0.20 mg×mL^{-1} of the TFA salt in PB-buffer. The baseline was recorded in the same conditions and subtracted manually during the post-processing. All the solutions were degassed under vacuum (~1 mbar) for 2 min just before the measurement. The final concentrations of the TFA salts were 100 µg×mL^{-1} in PB buffer (10 mM, pH 7.1). The cuvettes were washed successively with 1.0 M HCl (2 times), Milli-Q water (2 times) and PB-buffer (2 times) before each measurement.

The peptide dendrimer deconvolutions were performed on Dichroweb[2] using the data set 4 and 7 and the Contin-LL regression. This combination performed the best in the preliminary tests giving the most consistent results for the thermal denaturation of dendrimers and is in agreement with recommendations[3] for the method choice for short peptides.

Biofilm Formation Assay

Biofilms were grown on stainless steel coupons (grade 316L) under conditions similar to those described by Diggle et al.[4] Sterile steel coupons were placed at the edges of Petri dishes with 10 mL 0.05 % (v/v) nutrient broth containing the appropriate concentration of the test compound. Inocula of *P. aeruginosa* strains were prepared from 5 mL overnight cultures grown in LB broth. The optical density (600 nm) was corrected to 1 and aliquots of 100 µL were used to inoculate the medium contained within the Petri dishes. The Petri dishes were incubated at 37 ^{o}C on a rotary shaker (60 rpm) for 24 hrs. An additional 100 µL LB broth was added and the Petri dishes were incubated for a further 24 hrs. The test was performed in triplicate for each strain.

Staining and visualization of biofilms. The inoculated medium was removed from the Petri dish with a transfer pipette without disturbing the steel coupons.Sterile PBS (15 ml) was added and the dish agitated gently for 5 min to rinse the coupons. The PBS was removed and this rinse step was repeated, the coupons were dried and hest-fixed. The attached biofilms were then stained by applying 200 µl 0.1% (w/v) acridine orange for 2.5 min. After addition of 20 ml sterile PBS the dish was gently agitated as before for 5 min, the PBS was removed and the wash procedure repeated two further times. The coupons were air-dried and fixed to a glass microscope slide.
Biofilm attachment was visualized with an inverted fluorescent microscope (Nikon Eclipse TE 200) using the x10 objective lens and green filter. Ten images were collected per coupon with a JVC KY-F58 video camera. Sampling was conducted at random from each coupon, avoiding areas at the edges. With the “red” and “low” options set to the maximum level of 255, the area fraction for each image was calculated using the Lucia G/Comet software (Nikon UK) with a threshold set at between 175 and 185.

Biofilm dispersion assay

Biofilms were grown on stainless steel coupons (grade 316L) under conditions similar to those described by Diggle et al.[4] Sterile steel coupons were placed at the edges of Petri dishes with 10 mL 0.05 % (v/v) nutrient broth. Inocula of *P. aeruginosa* strains were prepared from 5 mL overnight cultures grown in LB broth. The optical density (600 nm) was corrected to 1 and aliquots of 100 µL were used to inoculate the medium contained within the Petri dishes. The Petri dishes were incubated at 37 ^{o}C on a rotary shaker (60 rpm) for 24 hrs. The inhibitors and additional 100 µl LB broth was added and the petri dishes were incubated for a further 24 h. The inoculated medium was removed from the Petri dish with a transfer pipette without disturbing the steel coupons and the coupons were rinsed with 10 ml 0.05% nutrient broth.. The remaining biofilm was visualized as described.

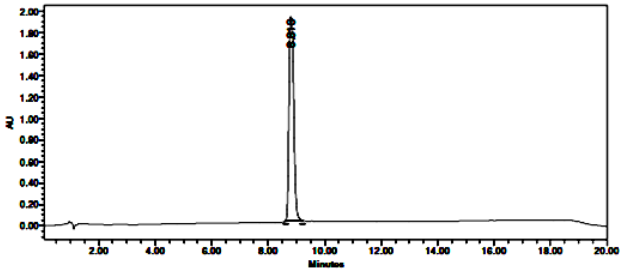
The biofilm formation and dispersion assays were performed with the same bacteria culture and batch of reagents for dendrimers **FD2**, D-**FD2**, **PA5**, **PA6** and monosaccharide controls.

Proteolysis Studies

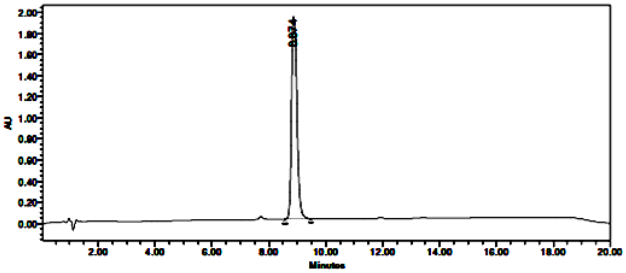
Test in solution with Trypsin and Chymotrypsin. The proteolysis was started by an addition of 5 µL of a freshly prepared stock solution of the protease (2.4 mg/mL of trypsin and 1.0 mg/mL of chymotrypsin) to 50 µL of dendrimer (2 mM in tris buffer (100 mM, pH 7.5)) and 45 µL of tris buffer (100 mM, pH 7.5). The reaction mixture was analysed after 10, 30, 60 min and 48 hours by RP-HPLC. Flowrate: 1.5 mL×min^{-1} (A/B 80/20 to 0/100 in 15 min). D-**FD2** was highly resistant to hydrolysis by both enzymes, even after 48 hrs, while **FD2** was cleaved in one hour. The fragments were identified by LC-MS. In the case of trypsin cleavage of the lysine-Ile peptidic bond gave a fragment with mw 1346 as one of the major peaks in the LC-MS (Figure S2). The cleavage sites for trypsin correspond to the acyl side of lysine residues although the presence of proline next to lysine in the dendritic structure made the cleavage at the LysPro site more difficult with the corresponding fragment cFucLys a minor peak in the LC-MS.Chymotrypsin cleaved the carboxy end of phenylalanine and leucine giving the expected four fragments (Figure S2). The L-lysine as branching unit did not give a cleavage site confirming the early study showing that the branching units was not very sensitive to proteases.

HPLC traces and LC-MS data for digestion experiments.

FD2 0 min

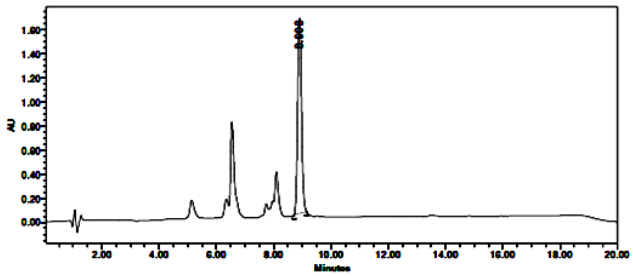


D-FD2 0 min

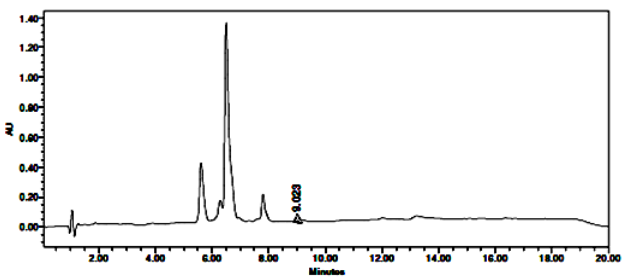


Digestion with trypsin:

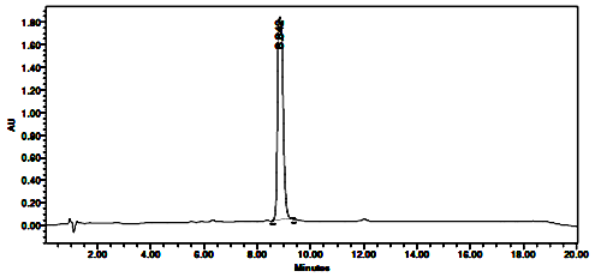
FD2 10 min



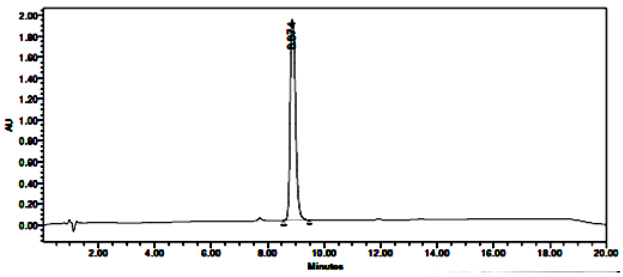
FD2 24 hrs



D-**FD2** 10 min



D-**FD2** 24 hrs



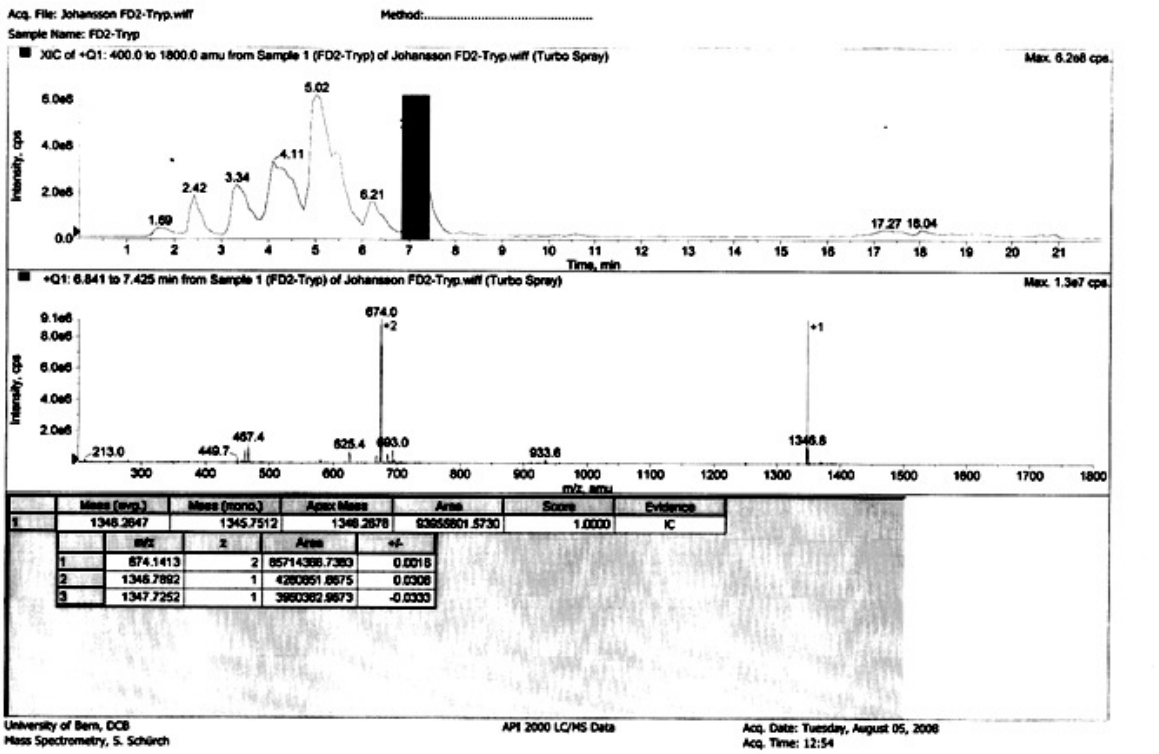
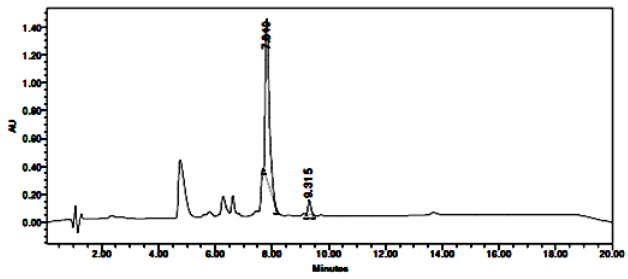


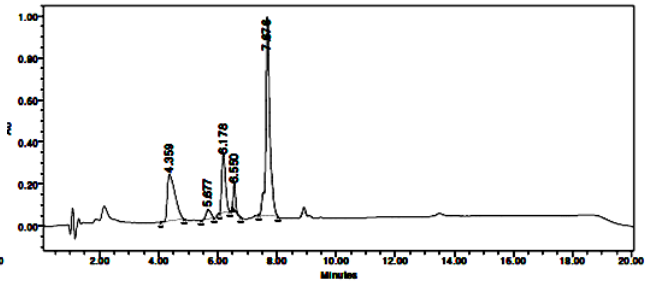
Figure S2. LC-MS of the Trypsin digestion of **FD2** after 24 h reaction.

Digestion with chymotrypsin:

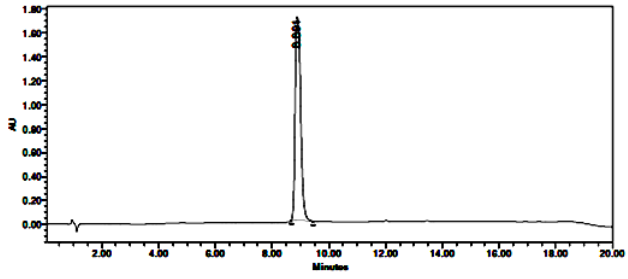
FD2 10 min



FD2 24 hrs



D-**FD2** 48 hrs



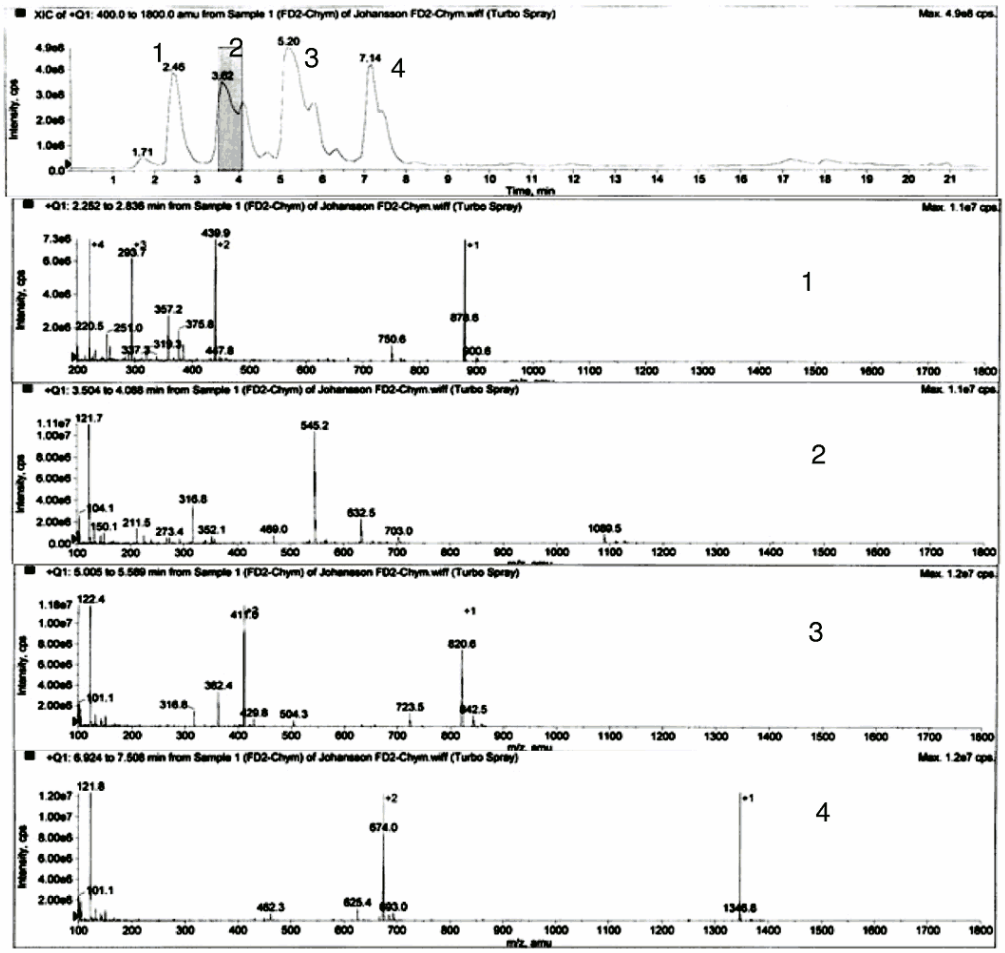


Figure S3 . The LC-MS trace for the hydrolysis of **FD2** in the presence of Chymoprypsin pH 7.5 after 24 h.. **1** = $(LysIle)_2LysHisIle$ (878); **2** = CFucLysProLeu (545); **3** = CFucLysProLeuLysPhe (820); **4** = $(CFucLysProLeu)_2LysPhe$ (1346).

Proteolytic stability test with human serum. Human serum (20 µL) to 20 µL of dendrimer solution, 2 mM in tris buffer (100 mM, pH 7.5). The proteolysis was stopped by addition of 100 µL MeOH (50 % TFA), filtered and then analysed by RP-HPLC. Flow rate: 1.5 $mL \times min^{-1}$ (A/B 80/20 to 0/100 in 15 min). Conversions were calculated by comparing integrals of starting material left after the test with a blank. The percentage of digestion was calculated from HPLC peak area (214 nm) with 4-hydroxy benzoic acid as internal reference.

Molecular Dynamic Simulations

Glycopeptide dendrimer structure building

The free dendrimers D-**FD2** $(CFuc\text{-}lys\text{-}pro\text{-}leu)_4(\textit{Lys}\text{-}phe\text{-}lys\text{-}leu)_2\textit{Lys}\text{-}his\text{-}leu\text{-}NH_2$ and **FD2** $(CFuc\text{-}Lys\text{-}Pro\text{-}Leu)_4(\textit{Lys}\text{-}Phe\text{-}Lys\text{-}Ile)_2\textit{Lys}\text{-}His\text{-}IleNH_2$ (*Lys* = branching lysine) were built starting as α-helix conformation using the peptide building dictionary interface in the Schrödinger suite maestro version 8.5. The parameters for the branching lysine residue were modified manually with the

correct dihedral angles and partial atomic charges derived from OPLS-AA (Optimized Potentials for Liquid Simulations-All Atom) [5] force field.

Macromodel Energy Optimizations

Built free dendrimers were optimized using default protocol in protein preparation wizard in maestro. The structures were subsequently minimized using macromodel (version 9.6) using the steepest decent method with maximum iterations of 500, a gradient convergence threshold of 0.05, and a constant dielectric with dielectric constant of 1. The potential force field used for minimizations protocol was OPLS-AA with extended cutoff values of (Van der Waals = 8.0; electrostatic = 20.0; H-bond =4.0).

System Preparation for Simulations

Molecular dynamics simulations were performed using OPLS-AA force field in desmond molecular dynamics system and maestro-desmond interoperability tool, version 2.0.[6] The systems were setup for explicit solvent simulations using desmond explicit molecular dynamic simulation program version 2.0. The solute was immersed in SPC water model[7] in cubic box spaced at 1nm from solute boundaries. The physiological salt concentration of 0.15 M was used.

Molecular dynamic simulations

The final parameterized structures were used for molecular dynamics simulation. Two independent simulations each of 10*ns* time period were performed using following three steps MD protocol: 1) Minimization 2) equilibration and 3) production run simulation.

*Minimization***.** The setup systems were minimized to remove close contact between solute-solvent molecules using the LBFGS method with maximum iterations of 200 cycles and convergence threshold (kcal/mol/Å) of 1.0. The step size was 0.005 Å and switch criteria was 25.0 kcal/mol/Å. The short range interactions used cutoff radius (Å) of 9.0, whereas the long range columbic interactions were taken into account using smooth Particle Mesh Ewald (PME) with Ewald tolerance of 1e-09.

Equilibration protocol. The equilibration protocol started with 2000 steps of steepest descent minimization of water molecules and ions to allow water molecules to assume a lower energetic geometry, whereas the solute was restrained with force constant of 50. The resulting systems were then subjected to 2000 steps of minimization with no restraints, reaching a root mean square gradient of 0.1 to assure the relaxation of the structures, followed by 12 ps of heating from 10 to 300 K in a constant volume ensemble with restraints on the solute heavy atom (50 kcal/mol $Å^2$)

followed by 12 ps of constant pressure without restraints at 300 K completed the equilibration step with convergences of energies, temperature, pressure, and density of the systems.

Productions protocol. The output co-ordinates from the equilibrated run were subsequently used in productions run for 10ns at 300K using NPT ensemble. The bond lengths to hydrogens were constrained with a variant of the M-SHAKE algorithm[8] To maintain 1 Atm at 300 K in NPT ensemble, the system was coupled to a Martyna-Tobias-Klein barostat[9], with relaxation time of 2ps with isotropic coupling style and a Nose-Hoover thermostat[10] (with relaxation time of 1ps). Long-range electrostatic interactions using a Particle Mesh Ewald method [11] and used a 64 × 64 × 64 Fourier space mesh with fifth-order B spline interpolation. The van der Waals (VDW) interactions and real space contributions to the electrostatics were truncated at 10 Å, and estimated the long-range VDW contributions to the energy and the pressure by assuming a homogeneous distribution of VDW spheres with dispersion coefficient 69.5 kcal/mol/Å. We used a RESPA integrator [12] with steps of 2 fs for bonded and short-range nonbonded interactions, and 6 fs for long-range electrostatics. During the course of simulations, quality checks were performed by monitoring total energy profile and heavy atom RMSD of structures in trajectories.

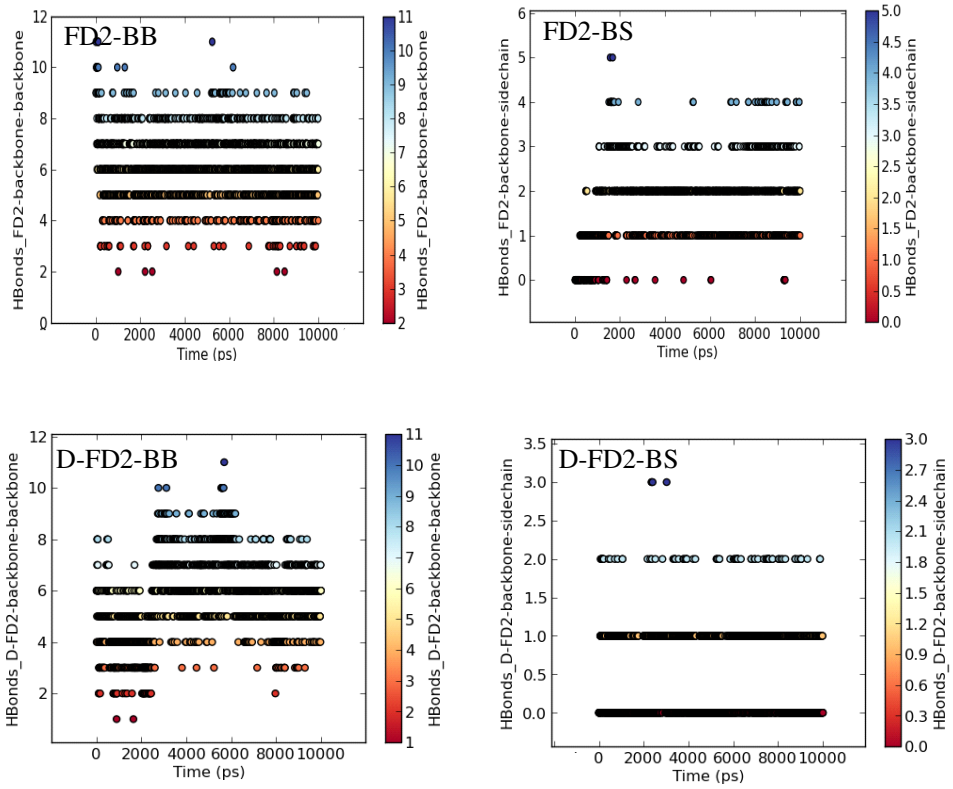


Figure S4. Intramolecular hydrogen bond analysis of **FD2** and **D-FD2** dendrimers: Data from 10ns of production run. Parameters used for defining Hbond: distance = 2.5Å; N-H..X (donor angle) > 120° and H..X-Y (acceptor angle) > 90°. **BB** (backbone-to-backbone) and **BS** (backbone-to-sidechain).

References

[1] E. M. V. Johansson, S. A. Crusz, E. Kolomiets, L. Buts, R. U. Kadam, M. Cacciarini, K. M. Bartels, S. P. Diggle, M. Camara, P. Williams, R. Loris, C. Nativi, F. Rosenau, K. E. Jaeger, T. Darbre and J. L. Reymond, *Chem. Biol.*, 2008, **15**, 1249-1257.

[2] Dichroweb 09/2008, http://www.cryst.bbk.ac.uk/cdweb/html/home.html.

[3] N. Sreerama, R. W. Woody, *Anal. Biochem*. **2000**, *287*, 252.

[4] S. P. Diggle, R. E. Stacey, C. Dodd, M. Camara, P. Williams and K. Winzer, *Environ. Microbiol.*, 2006, **8**, 1095-1104

[5] Jorgensen, W.L.; Maxwell, D.; Tirado-Rives, J. *J. Am. Chem. Soc*. **1996**, *118*, , 11225-11236.

[6]. Kevin J. Bowers, Edmond Chow, Huafeng Xu, Ron O. Dror, Michael P. Eastwood, Brent A. Gregersen, John L. Klepeis, István Kolossváry, Mark A. Moraes, Federico D. Sacerdoti, John K. Salmon, Yibing Shan, and David E. Shaw, "Scalable Algorithms for Molecular Dynamics Simulations on Commodity Clusters," Proceedings of the ACM/IEEE Conference on Supercomputing (SC06), New York, NY: ACM Press, 2006

[7] (a)Roberts, B.P.; Krippner, G.Y.; Scanlon, M. J.and Chalmers, D.K. *Macromolecules.* **2009**, *42*, 2784-2794; (b) Roberts, B. P.; Scanlon, M. J.; Krippner, G. Y.; Chalmers, D. K. *Macromolecules* **2009**, *42*, 2775-2783.

[8] Krautler, V.; Van Gunsteren, W. F.; Hunenberger, P. H. *J. Comp. Chem*. **2001**, 22, 501–508.

[9] Martyna, G. J.; Tobias, D. J.; and Klein, M. L. *J. Chem. Phys*. **1994,** *101*, 4177

[10] Oover, W. G. *Phys. Rev. A.* **1985**, *31*, 1695–1697.

[11] Darden, T. A.; York, D. M.; Pedersen, L. *J. Chem. Phys.* **1993**, *98*, 10089–10092.

[12] Tuckerman, M.; Martyna, G. J.; Berne, B. J. *J Chem Phys.* **1992**, *97*, 1990.