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The singular behavior of a β-type semi-synthetic two branches polypeptide. Three-dimensional structure and mode of action.

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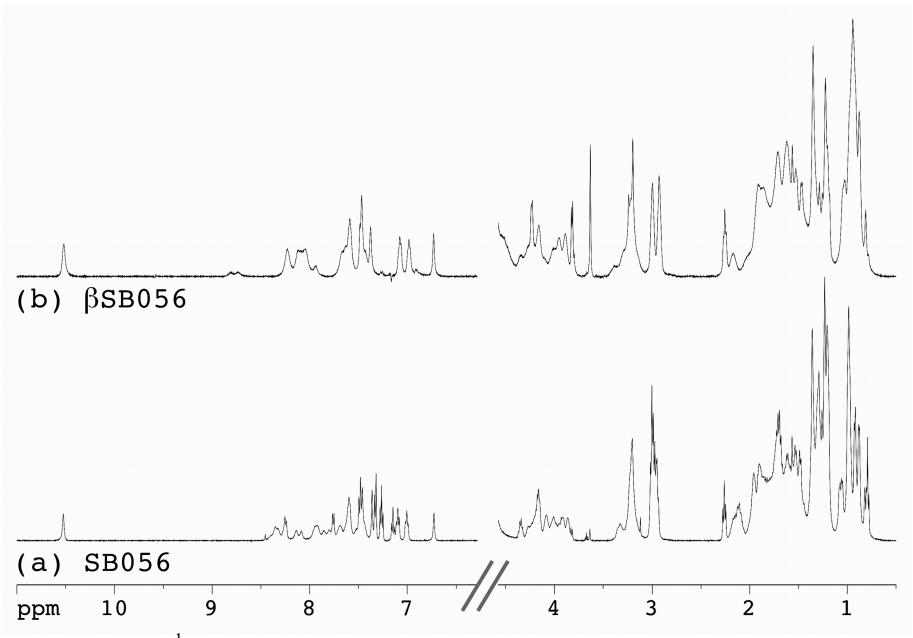

Figure S1. The ^{1}H spectrum is shown for **(a)** SB056 and **(b)** bSB056.

THE TWO REJECTED STRUCTURE MODELS

The so-called "sequential NOEs" are observed in two-dimensional NOESY spectra. These are the cross-peaks arising from the dipolar interaction between the two corresponding nuclei. NOEs are fundamental to determine the 3D structure of a peptide, since they allow sequential assignments (attributing the position along the sequence to the different amino acid spin systems) and because their relative intensity can be translated into inter-nuclear distance restraints for molecular modeling.[1] The case of SB056 and its analogue βSB056 is particularly challenging, due to the presence of two identical copies of the functional peptide unit. To distinguish intra- from inter-branch dipolar interactions is almost impossible, so that we decided to check all the possibilities without strictly following any preconfigured schemes for assignment. Three different hypotheses were put forward and cross-checked with MD simulations. Finally, only one structure model was found to be compatible with all the observations and, indeed, the only one being supported by MD, as reported in the main paper. Here the reader will found the details of the two rejected models.

THE FIRST STRUCTURE MODEL: HELIX-AND-COIL

As usually done for standard linear peptides, this model was obtained by looking first for short range NOEs (interactions between the i^{th} residue and residue i+1), and subsequently cross-checking the resulting attributions with the remaining NOEs. Figure S2 schematically reports the NOEs pattern obtained for the backbone proton resonances in the two cases, namely, SB056 and βSB056. In the case of the former, short range NOEs between the two alanine residues and the HNα and the

HNε of the lysine linker, respectively, allowed to distinguish the alpha- and the epsilon-branch. In particular, the alpha-branch appears to have a helical structure, thus, to be more regular and ordered than the other branch. In the case of βSB056, the same sequential NOEs were not as clearly observed and we preferred, thus, not to include them in the model. Nevertheless, one more and one less structured branch similarly resulted from NOEs assignments. We reported the alpha branch as the helical one by analogy with SB056. However, it is important to stress here, that the following discussion, as well as the main paper's discussion and conclusions, is independent of whether the alpha- or the epsilon- is the most structured branch.

Despite such different folding among the peptide branches would provide a possible explanation for the magnetic inequivalence of homologous residues on the two (see the main paper), such a huge difference is not plausible for numerous reasons.

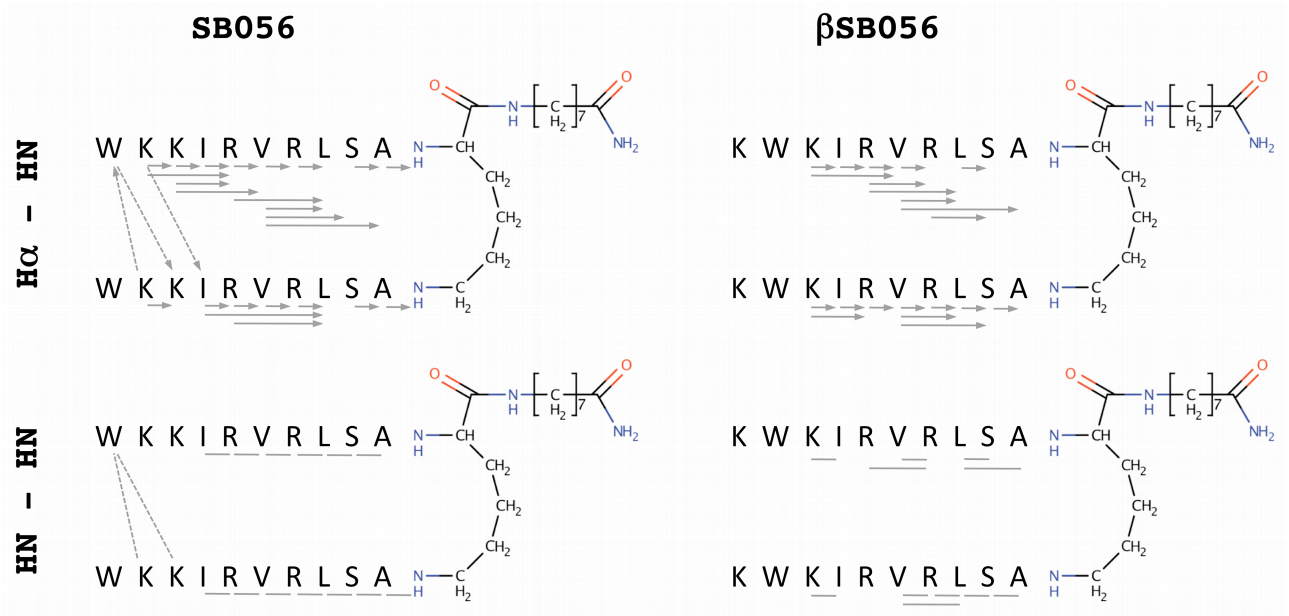

Figure S2. Attribution of backbone protons dipolar interactions leading to the first structural model referred to as 'helix and coil'. Results are shown for both SB056 (left) and its analogue βSB056 (right). In the case of Hα-HN interactions, arrows have been used instead of simple lines, where the tip indicates the amide proton involved. Dashed lines/arrows are used to indicate inter-branch interactions.

When these peptides were previously investigated in the absence of an anisotropic membrane mimicking model, they were found to have not a defined 3D structure.[2] The present model, thus, implies that the alpha-branch interacted with the micelle resulting in the helical folding, while the epsilon-branch was unbound and more exposed to the solvent. Such a scenario is questionable, at least, in the light of the identical amino acid sequence of the two branches. What might be the reason why the alpha-branch consistently binds to the micelle while the epsilon- branch doesn't? One should expect to have roughly one half of the peptides in the sample bound to the micelle through their alpha-branch, while the other half should be bound with the epsilon-branch, thus resulting, on average, in the two branches being not distinguishable at all. In addition, such a

structural difference between the two branches is not compatible with the J-couplings (see the main paper), that were comparable for homologous residues in the two branches and suggested a β-sheet like conformation. Finally, the alternate pattern of one hydrophobic / one hydrophilic residue that characterizes the sequence of the two branched peptides under investigation, is not suitable to form a helix on the micelle surface. Indeed, the resulting 3D structure for the helical branch would not be amphipathic and, as such, does not support a favorable interaction with the micelle surface.

Another interesting criticism against this helix-and-coil model is the presence of just few inter-branch NOEs at the N-termini in the case of SB056 (Figure S2). This would clearly show that the two branches are actually close each other, but why the two branches should come in close contact only with their N-termini? This is absolutely unlikely, since in the present case, the N-terminus is the peptide portion with the highest positive charge density, due to the presence of two lysine residues and the positively charged terminal amino group in each of the two branches.

A structure calculation was performed in vacuum through a simulated annealing procedure, in order to finally obtain the starting coordinates for MD simulations with explicit solvent and detergents molecules (see the main paper, methods section). Both the annealing and the MD were carried out applying the inter-proton distance restraints derived from the NOEs. Differently from the parallel hairpin model presented in the main paper, a strong interaction with the micelle surface, justifying the helical folding of the alpha-branch, was not observed. Figure S3 shows the last frame of the simulation as well as a plot of the residues average distance from the center of mass of the micelle. The peptide weakly interacted with the micelle surface by using just a few amino acid side chains. The helical folding of the alpha-branch appeared to be not very stable, with its backbone just giving a hint of such folded structure. Finally, numerous hydrophobic residues were unfavorably exposed to solvent contacts. The average distance analysis (Figure S3b) clearly showed that the peptide was almost completely protruded outside the micelle, providing no support to the helix-and-coil model.

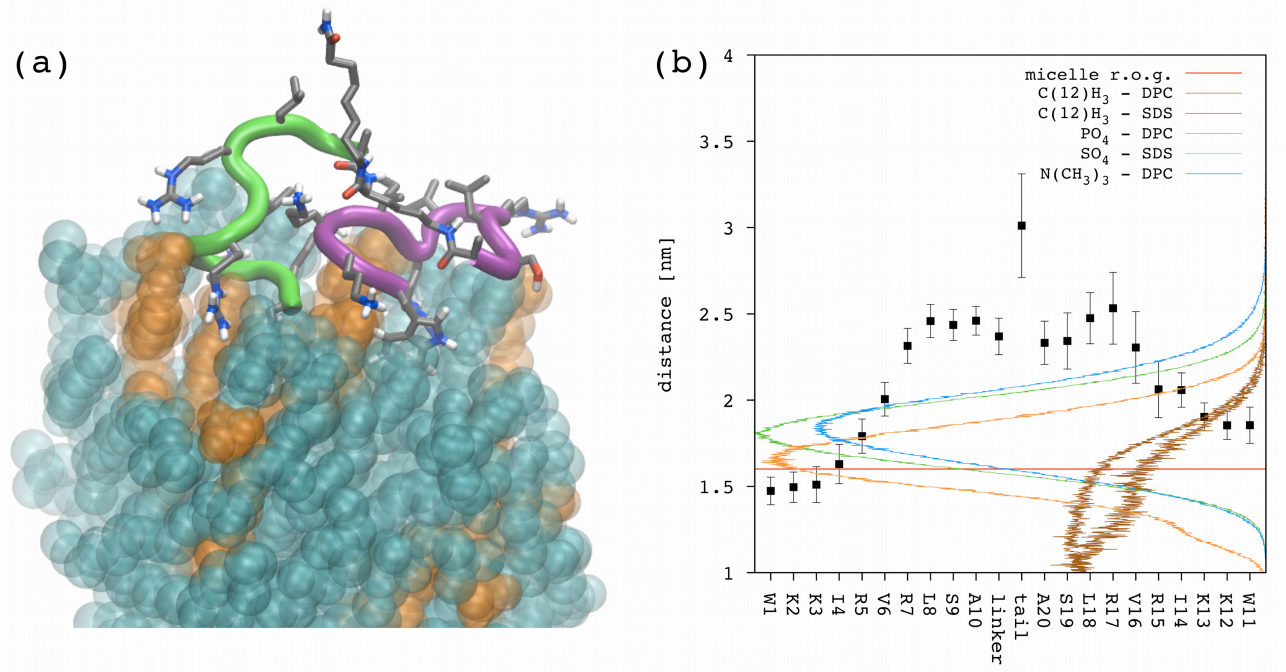

Figure S3. The helix-and-coil hypothesis. The last frame of the MD simulation performed on SB056 in the presence of a DPC/SDS micelle is shown in **(a)**. The atoms of the detergents are represented with the corresponding van der Waals spheres. DPC and SDS are colored in cyan and orange, respectively. The backbone of the alpha- and epsilon-branch are represented with a differently colored trace, green and purple, respectively. The average distance with error bars of residues' backbone from the center of mass of the micelle is plotted in **(b)** together with the radial distribution function computed for different chemical groups of the two detergents. The horizontal red line is used to indicate the radius of gyration of the micelle.

THE SECOND STRUCTURE MODEL: A DIMER

Another possible interpretation of the observed NOEs was obtained by preserving the short range i,i+1 contacts and by assigning the remaining NOEs as inter-molecular interactions. Figure S4 shows the sequential assignments. In practice, this approach led to the hypothesis of a dimer on the micelle surface, characterized by the parallel pairing of the alpha-branch of two distinct SB056 peptides. For the sake of completeness, it has to be mentioned that also an antiparallel pairing was checked but found to be absolutely not compatible with the observed NOEs. Only the parallel orientation of the two SB056 molecules allowed us to obtain a consistent assignment of the NOEs.

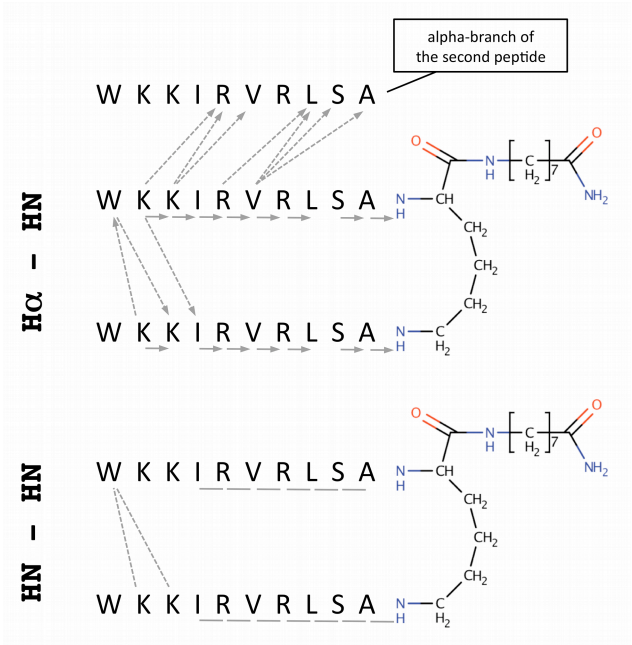

Figure S4. Attribution of backbone protons dipolar interactions leading to the second structural model referred to as 'dimer'. Results are shown for SB056. In the case of Hα-HN interactions, arrows have been used instead of simple lines, where the tip indicates the amide proton involved. Dashed lines/arrows are used to indicate inter-branch interactions.

Despite such a partial β-type structure might provide a valid explanation for the J-coupling constants of ~10 Hz (see the main paper), at least for the alpha-branch, almost all the drawbacks of the helix-and-coil model are not removed. The hypothesized dimer implies that the two peptide branches are extremely different, which is not compatible with the comparable J-coupling values observed for homologous residues located in the two branches. The alpha-branch appears to have a more regular and stable structure than the epsilon-branch, without any reasonable physical explanation. Why should the homodimer be formed only by alpha-branch pairing? One should expect also the formation of epsilon-epsilon and alpha-epsilon homodimers, resulting in the two branches being not distinguishable at all. At the same time, the few inter-branch medium range interactions involving the first residues rise the same concerns discussed in the case of the helix-and-coil hypothesis. In addition, in the light of the [L]/[P] ratio employed (i.e. 50) and taking into account that a DPC/SDS micelle is formed by roughly 50-70 detergent molecules,[3] there was one peptide per micelle in solution, thus, the probability that two SB056 molecules bound the same micelle was rather low. Finally, it can be noted that the two interactions present in the epsilon-branch of the helix-and-coil hypothesis (Figure S2) were not included in this dimer model because they would have imply a close contact also between the two epsilon-branches, making even more evident that the dimer hypothesis is highly questionable.

Nevertheless, the structure calculations were performed. Both the annealing and the MD were carried out applying the inter-proton distance restraints derived from the NOEs attribution. The interaction with the micelle was even weaker than for the helix-and-coil model. Figure S5 shows the last frame of the simulation as well as a plot of the residues average distance from the center of mass of the micelle for both the peptides molecules of the homodimer. The dimer was entirely exposed to the solvent and extremely flexible, showing the inconsistency of the present model.

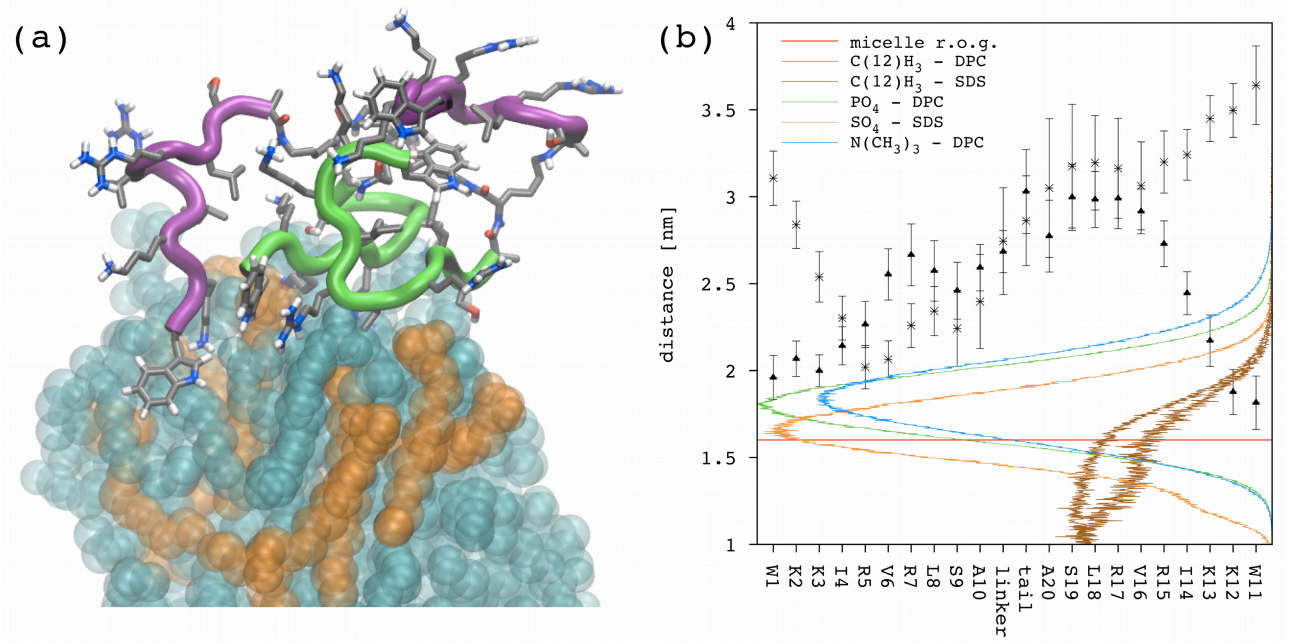

Figure S5. The dimer hypothesis. The last frame of the MD simulation performed on SB056 in the presence of a DPC/SDS micelle is shown in **(a)**. The atoms of the detergents are represented with the corresponding van der Waals spheres. DPC and SDS are colored in cyan and orange, respectively. The backbone of the alpha- and epsilon-branch are represented with a differently colored trace, green and purple, respectively. The average distance with error bars of residues' backbone from the center of mass of the micelle is plotted in **(b)** for both the peptides forming the homodimer (triangles and stars, respectively), together with the radial distribution function computed for different chemical groups of the two detergents. The horizontal red line is used to indicate the radius of gyration of the micelle.

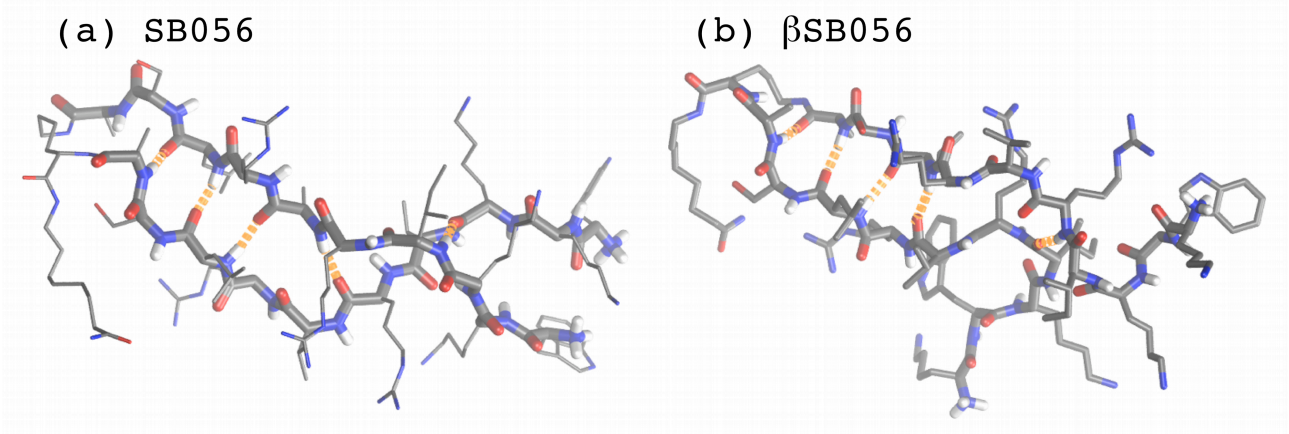

Figure S6. One of the most visited conformation is shown for **(a)** SB056 and **(b)** bSB056. Intramolecular inter-branches Hbonds are shown with dashed orange lines. Micelle and water molecules are not shown for the sake of clarity.

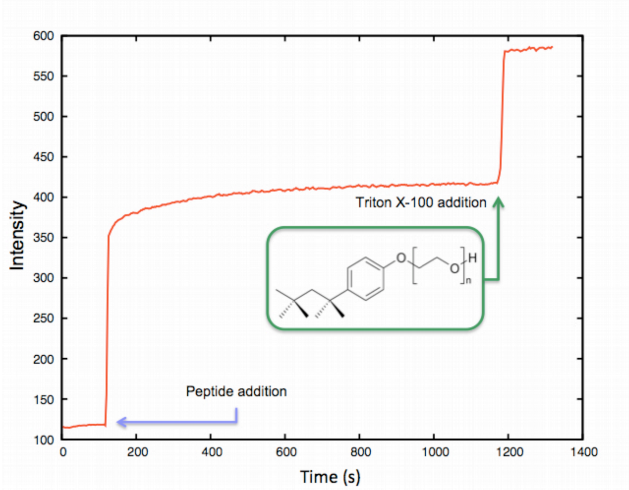

Figure S7. Typical calcein release kinetics recorded in this work, despite the peptide and the vesicles employed. The only difference form experiments to experiments was the relative level of release, depending upon the [P]/[L] (see figure 7 in the main paper).

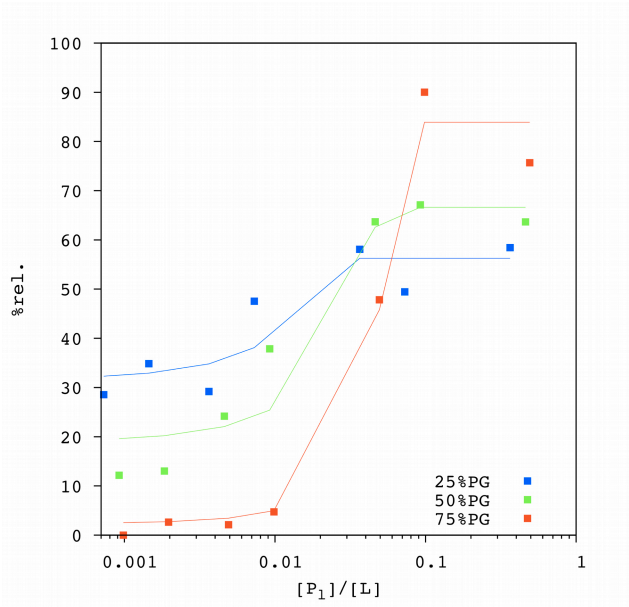

Figure S8. Results of the calcein leakage experiments are shown for βSB056 (the same as in figure 7a of the main paper). The x-axis has been transformed into $[P_t]/[L]$. Experimental points are shown together with the calculated data (solid lines) obtained by applying the all-or-none model described in the methods. In figure 7a of the main paper the latter was applied considering only the outer leaflet to estimate the electroneutrality conditions (see the main text). Here, the results obtained by considering both leaflets are shown.

MINIMAL INHIBITORY CONCENTRATION ASSAY

PROCEDURE

Minimal inhibitory concentration (MIC) of βSB056 and βSB056-NT (exactly the same peptide without the aminooctanamide tail $(KWKIRVRLSA)_2$-K-NH_2) against two Gram-negative (*E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853) and one Gram-positive (*S. aureus* ATCC 25923) bacteria was determined following a microdilution assay in 96-well plates, according to CLSI guidelines.[4] Due to the relatively low solubility in water, peptides were firstly solubilized in chloroform/methanol (1/1 V/V). Then, proper aliquots were taken in order to prepare the different solutions at the exact concentration needed for the assay. The organic solvent was evaporated under a gentle stream of nitrogen, followed by vacuum overnight. The peptide film was finally resuspended in a solution of Mueller-Hinton (MH) broth + 10% DMSO. Bacteria were grown in Luria-Bertani (LB) medium at 37°C till a mid-log-phase (OD_{590} equal to 0.8) and diluted to a final titer of $2x10^6$ CFU/mL with MH before adding 50 µL of suspension to the test plate (final volume 100 µL). The final concentration of DMSO in the plate was 5%. A sterility control of the media used was added in the plate. A growth control was also present in order to exclude any significant effect of DMSO on bacteria growth. The plates were incubated at 37°C for 16-18 h. In order to better observe bacteria growth, 20 µL of resazurin solution (0.2 g/L) was added to each well, before incubating the plates for additional 2h. The resazurin dye turns from blue to pink after its reduction due to bacterial growth.[5] The MIC was defined as the minimal peptide concentration where no bacterial growth was visible.

RESULTS AND DISCUSSION

Either paramagnetic relaxation enhancement NMR experiments and MD simulations revealed that the aminoctanamide tail of both SB056 and βSB056 peptides was completely exposed to the solvent (figure 5). From these results it was hard to think that the tail was really fundamental for the peptides' activity. In order to verify this point, the antimicrobial activity of βSB056 against some reference bacteria strains was compared to the one of a peptide with identical sequence but without the tail (βSB056-NT, $[KWKIRVRLSA]_2$-K-NH_2). In particular, the MIC values were determined for both peptides against two gram-negative (*E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853) and one gram-positive (*S. aureus* ATCC 25923) bacteria. Results are summarized in table S3. Antimicrobial activity of the two peptides was absolutely comparable against all the three tested

strains, with the difference always within one dilution. These findings are in perfect agreement with the results from NMR and MD, bolstering the conclusion that the activity is due to the physico-chemical properties of the peptide sequence and the dendrimeric architecture but it is not absolutely modified by the aminooctanamide tail.

References

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Tables

Table S1. ^{1}H and ^{13}C resonance assignments for SB056 in the presence of mixed micelles formed by DPC and SDS at 3:1 molar ratio in phosphate buffer saline at pH 7.4. The peptide to detergents molar ratio was 1:50. For each position along the amino acid sequence, two series of assignments are orderly reported for each resonance. Values corresponding to the alpha- and the epsilon- branch are reported on the left and the right, respectively.

residue	^{1}H [ppm]									^{13}C [ppm]				$^{3}J_{HNH\alpha}$	
	HN		Hα		Hβ		others			Cα		Cβ			
W	8.246	---	4.603	4.098	3.215 2.999	3.343 3.201	HN1 2H 4H 5H 6H 7H	10.525 7.362 7.322 7.141 7.255 7.327	10.525 7.362 7.598 7.004 7.098 7.488	57.517	57.251	39.451	---	7.06	---
K	---	7.468	4.079	4.348	1.776 ---	1.781 1.634	γ δ ε HNζ	1.275 1.626 2.948 ---	1.363 1.691 2.969 ---	57.760	55.322	32.612	32.869	---	7.17
K	8.392	7.760	4.216	4.169	1.904 1.833	1.837 1.734	γ' γ'' δ ε HNζ	1.551 1.460 1.626 2.948 ---	1.359 --- 1.722 2.990 ---	57.760	57.271	32.008	33.711	---	6.824
I	7.951	7.931	3.865	3.918	2.113	2.095	γ1' γ1'' γ2 δ	1.497 --- 1.270 ---	1.682 1.304 0.994 0.975	64.474	65.082	37.620	37.620	7.51	6.82
R	8.350	8.321	3.957	4.024	1.988 1.898	1.983 1.882	γ δ HNε HNη	1.687 3.220 --- ---	1.696 3.220 --- ---	59.465	---	29.884	29.884	6.90	10.51
V	7.699	7.681	3.866	3.910	2.173	2.163	γ1 γ2	1.079 1.010	1.053 0.978	63.623	63.128	---	31.862	10.240	9.890
R	7.811	7.854	4.243	4.257	1.951	1.951	γ δ HNε HNη	1.756 3.214 --- ---	1.756 3.214 --- ---	61.301	---	35.413	35.011	---	---
L	8.261	8.136	4.160	4.175	1.590	1.590	γ δ1 δ2	1.879 0.921 0.880	1.879 0.921 0.880	57.271	57.251	27.727	27.727	10.39	10.27
S	8.085	7.911	4.151	4.204	4.033 3.994	4.021 3.992				61.123	60.259	63.006	63.006	10.10	10.24
A	7.597	7.524	4.271	4.342	1.531	1.482				53.490	52.393	18.692	19.418	---	10.240
linker	7.628		4.170		1.849		γ δ ε' ε'' HNζ	--- --- 3.308 3.160 7.584		57.251		---		---	
tail							HN8 HC8 HC7 HC6 HC5 HC4 HC3 HC2 HN1' HN1''	7.437 3.204 1.548 1.350 1.350 1.350 1.607 2.260 7.462 6.724							

Table S2. ^{1}H and ^{13}C resonance assignments for βSB056 in the presence of mixed micelles formed by DPC and SDS at 3:1 molar ratio in phosphate buffer saline at pH 7.4. The peptide to detergents molar ratio was 1:50. For each position along the amino acid sequence, two series of assignments are orderly reported for each resonance. Values corresponding to the alpha- and the epsilon- branch are reported on the left and the right, respectively.

residue	^{1}H [ppm] HN		Hα		Hβ		others			^{13}C [ppm] Cα		Cβ		$^{3}J_{HNH\alpha}$	
K	---	---	3.898	---	1.740	---	γ	1.335	---	56.091	---	34.048	---	---	---
							δ	1.629	---						
							ε	2.926	---						
							HNζ	---	---						
W	---	---	4.622	---	3.386	---	HN1	10.519	---	---	---	---	---	---	---
					3.297	---	2H	7.373	---						
							4H	7.589	---						
							5H	6.982	---						
							6H	7.076	---						
							7H	7.476	---						
K	8.806	8.740	4.355	4.356	1.854	1.918	γ	1.429	1.431	57.002	57.002	32.095	32.095	a	a
							δ	1.703	1.715						
							ε	2.999	2.999						
							HNζ	---	---						
I	8.125	8.037	3.894	3.954	1.926	1.907	γ1’	1.627	1.627	---	---	37.934	37.934	a	a
							γ1’’	1.262	1.262						
							γ2	0.945	0.945						
							δ	0.936	0.936						
R	8.246	8.245	4.027	4.120	1.918	1.894	γ	1.715	1.687	---	---	30.261	30.261	a	a
							δ	3.223	3.226						
							HNε	---	---						
							HNη	---	---						
V	7.678	7.670	3.877	3.945	2.177	2.156	γ1	1.047	1.023	64.913	64.099	32.112	32.212	a	a
							γ2	0.987	0.973						
R	8.047	8.069	4.252	4.297	1.930	1.914	γ	1.794	1.687	60.198	---	30.261	30.261	a	a
							δ	3.212	3.226						
							HNε	---	---						
							HNη	---	---						
L	8.095	8.125	4.163	4.178	1.606	1.607	γ	1.859	1.859	57.391	57.391	28.565	27.811	a	a
							δ1	0.913	0.913						
							δ2	0.843	0.874						
S	8.217	7.938	4.164	4.211	4.040	3.981				61.148	61.451	63.178	63.351	a	a
A	7.637	7.589	4.270	4.336	1.520	1.468				53.677	52.554	19.013	19.713	a	a
linker	7.632		4.167		1.819		γ	---		56.700		33.774		---	
							δ	---							
							ε	3.301							
							HNζ	7.596							
tail							HN8	7.427							
							HC8	3.198							
							HC7	---							
							HC6	1.361							
							HC5	1.361							
							HC4	1.361							
							HC3	1.599							
							HC2	2.258							
							HN1’	7.462							
							HN1’’	6.734							

a These J couplings (measured from DQF-COSY cross-peaks splitting) were found to be larger than 10 Hz. By taking into account possible overestimation due to a slight signal broadening with respect to the case of SB056, the important point is that all of these J couplings were significantly larger then 8 Hz.

Table S3. MIC values of βSB056 and βSB056-NT against reference gram-negative and positive strains.

MIC (μM)	**Microorganism and strain**		
	E. coli ATCC 25922	*P. aeruginosa* ATCC 27853	*S. aureus* ATCC 25923
βSB056	25	50	>50
βSB056-NT	50	25	50