# A Molecular Umbrella Approach to the Intracellular Delivery of siRNA

**Lauren L. Cline**[†], **Vaclav Janout**[†], **Michael Fisher**[≠], **Rudolph L. Juliano**[≠], and **Steven L. Regen**[*,†]

[†]Department of Chemistry, Lehigh University, Bethlehem, Pennsylvania 18015

[≠]Division of Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, North Carolina

## Abstract

A series of diwalled and tetrawalled molecular umbrellas have been synthesized using cholic acid, spermidine, and lysine as starting material. Coupling of these molecular umbrellas to an octaarginine peptide afforded agents that were capable of promoting the transport of small interfering RNA (siRNA) to HeLa cells, as judged by the knockdown of enhanced green fluorescent protein (eGFP) expression. The efficiency of this knockdown was found to increase with an increasing number of facially amphiphilic walls present, and also when a cleavable disulfide linker was replaced with a non-cleavable, maleimido moiety. The knockdown efficiency that was observed for one tetrawalled molecular umbrella-octaargine conjugate was comparable to that observed with a commercially available transfection agent, Lipofectamine 2000, but showed less cytotoxicity.

---

The ability of small interfering RNA (siRNA) to silence gene expression has generated considerable interest in recent years because of their potential as chemotherapeutic agents and as biological tools.[1–4] One of the major goals in this area has been to improve the delivery of siRNA directly to the cytosol in order to allow for more efficient incorporation into the RNA-induced silencing complex (RISC)—a key step in the silencing process.[2] To date, most approaches that have been used to deliver siRNA appear to rely on endocytotic pathways. Because endosomal release is known to be inefficient, a more direct means of delivery would be highly desireable. In particular, if siRNA could be transported into the cytosol *via* passive diffusion, significant improvement in their efficacy should be possible. With this ultimate goal in mind, we have begun to explore *molecular umbrellas* as delivery agents for siRNA.[5] Here, we report our results with first-generation molecular umbrellas bearing a pendant octaaarginine peptide moiety for binding to siRNA.

As discussed elsewhere, molecular umbrellas are conjugates composed of two or more facial amphiphiles (i.e., "walls") that are attached to a central scaffold.[5] When immersed in a hydrophilic environment, these molecules create a hydrophilic exterior. Conversely, when immersed in a hydrophobic environment, they create a hydrophobic exterior. Recently, we have shown that the transport properties of molecular umbrellas in model membranes *do not follow the classic size/lipophilicity rule*.[6] Specifically, bilayer transport rates were found to increase with increasing numbers of umbrella walls and increasing facial hydrophilicity. This is exactly the opposite of what is expected based on existing drug transport theory.[7] In

slr0@lehigh.edu.

addition, we and other researchers have shown that molecular umbrellas can readily enter live HeLa cells and that passive transport may play a significant role in this entry.[8,9] Based on these features, molecular umbrellas offer an opportunity for promoting the passive transport of biologically-active agents across cell membranes in ways that have not previously been possible.

As a first step in exploring molecular umbrellas for siRNA delivery, we examined a series of molecular umbrella-octaarginine conjugates for their ability to reduce (i.e., "knockdown") gene expression of enhanced green fluorescent protein (eGFP) in HeLa cells. Octaarginine was chosen because of its contguous array of positive charges for binding to siRNA through electrostatic interactions and hydrogen bonding. Our working hypothesis was that such conjugates would create a "slide" within a plasma membrane for the passage of siRNA and/ or a sheath that shields the siRNA as it crosses the bilayer (Figure 1). A related mechanism that can also be envisioned is one in which the conjugates act like "ferries" to shuttle the cargo across the membranes (not shown). In the present study, our primary aim was four-fold: (i) to determine whether molecular umbrella-octaarginine conjugates canpromote siRNA-induced knockdown of eGFP expression beyond that of octaarginine itself, (ii) to test whether knockdown efficiency is dependent on the size of the umbrella that is used, (iii) to determine whether the introduction of a cleavable linker in a molecular umbrella-octaarginine conjugate can improve knockdown efficiency, and (iv) to judge the potential importance of a "slide" versus a "sheath" mechanism of delivery.[2]

With this purpose in mind, five agents were chosen as synthetic targets for this work; that is, conjugates **1**, **2**, **3**, **4** and **5** (Figure 2). Thus, comparison of **1** with **2**, and **3** with **4**, addresses the question of how umbrella size may influence siRNA delivery. Comparison of **1** with **3**, and **2** with **4**, bearing a cleavable disulfide or a non-cleavable maleimido moiety for conjugation, addresses the question of conjugate lability on knockdown efficiency. As a control for investigating the role that only the peptide portion of these conjugates play in siRNA delivery, we synthesized an octaarginine analog (**5**), which contains a 2-mercaptoethanol moiety instead of a molecular umbrella. To place the knockdown efficiencies of these agents into perspective, we have compared them with that found using Lipofectamine 2000—a proprietary formulation that is widely used as a "gold standard" for transfection.

The synthetic method that was used to prepare conjugate **1** is shown in Scheme 1. In brief, Boc-protection of the terminal amino groups of spermidine with 2-(Boc-oxyimino)-2-phenylacetonitirile) (Boc-ON) to give **6**, followed by acylation with *N*-[O-1,2,3-benzotriazin-4(3H)one-yl]-3,(2-pyridyldithio)propionate (BPDP), afforded **7**.[10] Subsequent deprotection to give **8**, followed by acylation with the N-hydroxysuccinimide ester of cholic acid (Ch-NHS) afforded **9**, which was then reacted with the free thiol form of **5** (designated as **Pep**-SH) to give **1**. The synthesis of **2** was carried out in a similar manner (Scheme 2). In this case, lysine dicholamide was first activated with N,N,N′,N′-tetramethyl-O-(N-succinimidyl) uranium tetrafluoro borate (TSU) and then used to acylate **8** to give **10**.[11] Conjugate **4** was synthesized by first acylating both terminal amino groups of spermidine with lysine dicholamide, followed by acylation of its secondary amine with 3-maleimido-propanic acid and conjugated addition of **Pep**-SH (Scheme 3). The analogous diwalled conjugate, **3**, was synthesized by a similar route (note shown). Finally, the control peptide, **5**, was obtained by reacting **Pep**-SH with 2-(2-pyridyldithio)ethanol (not shown).

Using procedures described in the Supporting Information section and 50 nM eGFP siRNA, knockdown efficiencies were determined with concentrations of **1**, **2**, **3**, **4** and **5** that varied from 100 to 2000 nM. In control experiments, where HeLa cells were treated only with eGFP siRNA, or with eGFP siRNA plus **5**, no significant knockdown was observed (Figure

conjugate), **4** exhibited no significant decrease in cell viability, whereas the use of Lipofectamine 2000 resulted in a moderate decrease in cell viability.

In an effort to judge the potential importance of a slide versus a sheath mechanism of transport, knockdown experiments were carried out with **4** in two different ways; that is, (i) by first incubating HeLa cells with the conjugate *prior to the addition* of siRNA, and (ii) incubating **4** with the siRNA followed by incubation with the HeLa cells. As shown in Figure 5, the latter (where the umbrella conjugate is premixed with the eGFP siRNA) clearly results in a greater knockdown efficiency. These results suggest that contributions from a sheath mechanism of delivery are likely to be more important than a slide mechanism (Figure 5).

The present findings demonstrate the feasibility of applying molecular umbrella chemistry to siRNA transport. They also show that significant improvements are possible in the case of molecular umbrella-octaargine conjugates by increasing the number of umbrella walls and increasing the stability of the linker used to connect both components. The fact that the knockdown efficiency of **4** is at a level that is comparable to Lipofectamine 2000 while exhibiting reduced cytotoxicity provides considerable incentive for exploring molecular umbrellas more broadly in this context. It should be noted, in this regard, that this comparison is based on experiments in which HeLa cells were transfected for 4 h with the siRNA complexes in *serum-free media prior* to changing to serum-containing media. When serum was present throughout the entire course of the transfection, the activity of **4** was reduced by ca. 50% (Supporting Information). In contrast, serum had a negligible effect on the activity of Lipofectamine 2000. Whether further changes in the composition and structure of such molecular umbrella conjugates can minimize such reduction in activity remains to be established.

Efforts currently in progress are focused on (i) the synthesis of related conjugates in which the sense strand of a double-stranded siRNA has been covalently attached to the umbrella of a molecular umbrella, thereby circumventing the need for a pendant octaarginine moiety, and (ii) gaining insight into the probable contributions made from passive transport and endocytotic pathways for cellular entry. The results of these studies will be reported in due course.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

## References

1. Stanton MG, Colletti SL. Medicinal chemistry of siRNA delivery. J Med Chem. 2010; 53:7887–7901. [PubMed: 20806941]

2. Juliano R, Alam RM, Dixit V, Kang H. Mechanisms and strategies for effective delivery of antisense and siRNA oligonucleotides. Nucleic Acids Res. 2008; 36:4158–4171. [PubMed: 18558618]

3. Wolfrum C, Shi S, Jayaprakash KN, Jayaraman M, Wang G, Pandey RK, Rajeev KG, Nakayama T, Charrise K, Ndungo EM, Zimmermann T, Koteliansky V, Manoharan M, Stoffel M. Mechanisms and optimization of in vivo delivery of lipophilic siRNAs. Nature Biotech. 2007; 25:1149–1157.

4. Mahon KP, Love KT, Whitehead KA, Qin J, Akinc A, Leshchiner E, Leshchiner I, Langer R, Anderson DG. Combinatorial approach to determine functional group effects on lipidoid-mediated siRNA delivery. Bioconjugate, Chem. 2010; 21:1448–1454.

5. Janout V, Regen SL. Bioconjugate-based molecular umbrellas. Bioconjug, Chem. 2009; 20:183–192. [PubMed: 19053303]

6. Mehiri M, Chen WH, Janout V, Regen SL. Molecular umbrella transport: exceptions to the classic size/lipophilicity rule. J Am Chem Soc. 2009; 131:1338–1339. [PubMed: 19140686]

7. Pidgeon C, Ong S, Liu H, Qiu X, Pidgeon M, Dantzig AH, Munroe J, Homback WJ, Kasher JS, Glunz L, Szczerba T. IAM chromatography: an in vitro screen for predicting drug permeability. J Med Chem. 1995; 38:590–594. [PubMed: 7861406]

8. Mehiri M, Jing B, Ringhoff D, Janout V, Cassimeris L, Regen SL. Cellular entry and nuclear targeting by a highly anionic molecular umbrella. Bioconjugate Chem. 2008; 19:1510–1513.

9. Ge D, Wu D, Wang Z, Shi W, Wu T, Zhang A, Hong S, Wang J, Zhang Y, Ren L. Cellular uptake mechanism of molecular umbrella. Bioconjugate Chem. 2009; 20:2311–2316.

10. Janout V, Lanier M, Regen SL. *N*-[O-1,2,3-Benzotriazin-4(3H)one-yl]-3,(2-pyridyldithio)propionate: a more reactive alternative to SPDP. Tetrahedron Lett. 1999; 40:1107–1108.

11. Chen WH, Shao XB, Regen SL. Poly(choloyl)-based amphiphiles as pore-forming agents: transport-active monomers by design. J Am Chem Soc. 2005; 127:12727–12735. [PubMed: 16144423]

## Synopsis

A series of diwalled and tetrawalled molecular umbrella-octaarginine conjugates have been found capable of transporting siRNA into HeLa cells, as judged by the knockdown of green fluorescent protein. A tetrawalled conjugate, containing a non-cleavable maleimido linkage, exhibited an efficiency that was comparable to that of Lipofectamine 2000 but with reduced cytotoxicity.
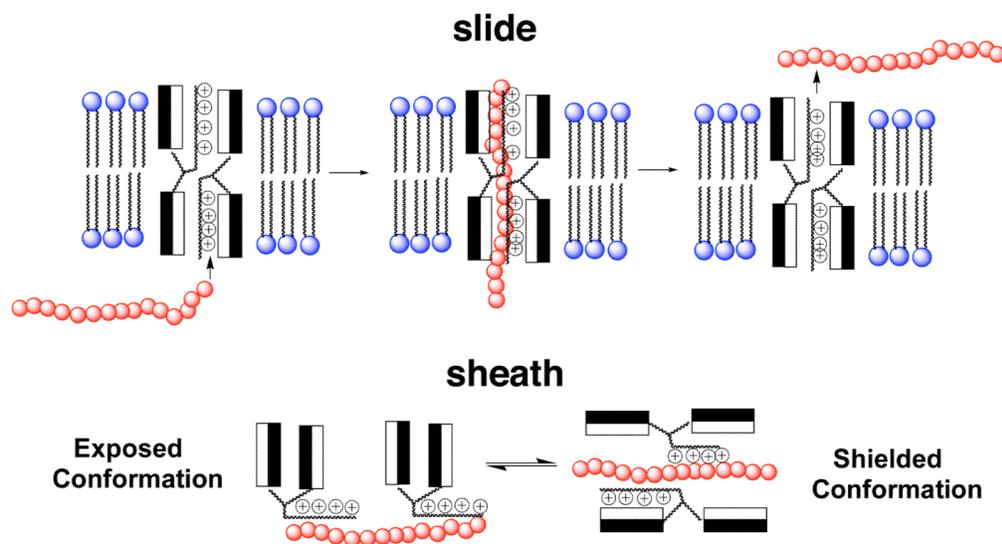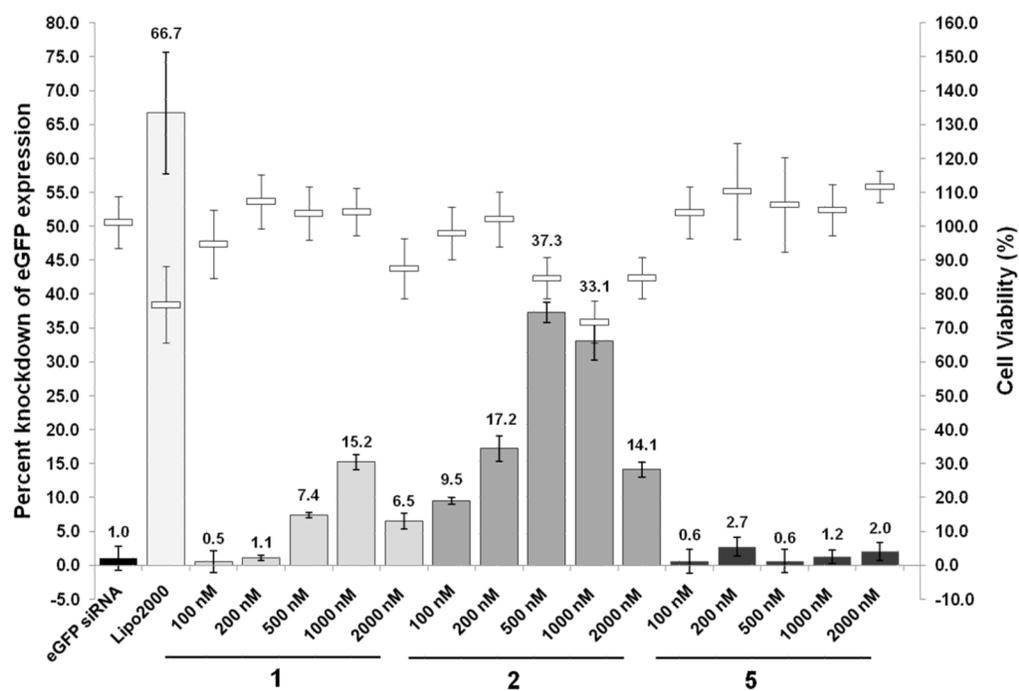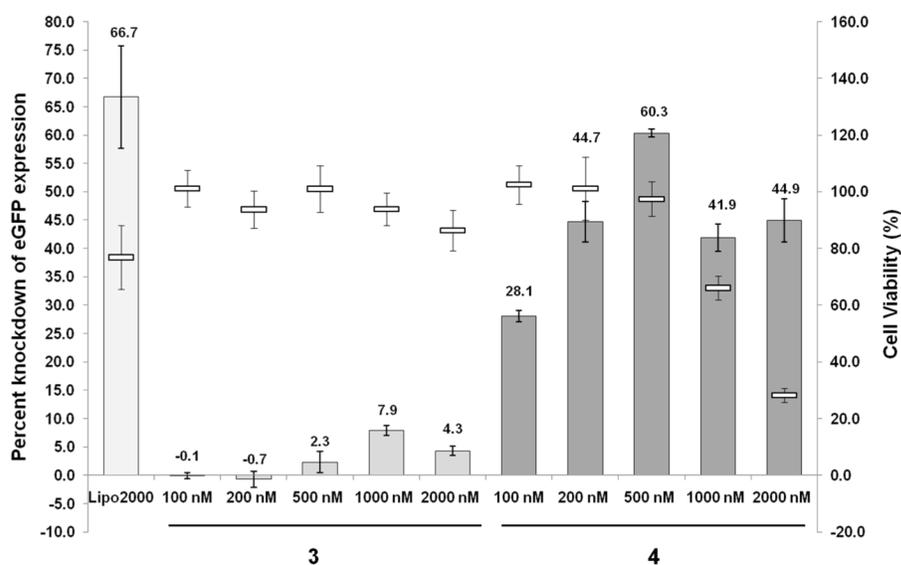
**Figure 1.**
A hypothetical "slide" created by two diwalled molecular umbrella-oligoarginine conjugates inserting into a plasma membrane (top) versus a sheath (bottom), affording an exposed or shielded conformation. The black and white rectangles represent lipophilic and hydrophilic faces of the umbrella, respectively, and the red spheres represent individual units of the siRNA.
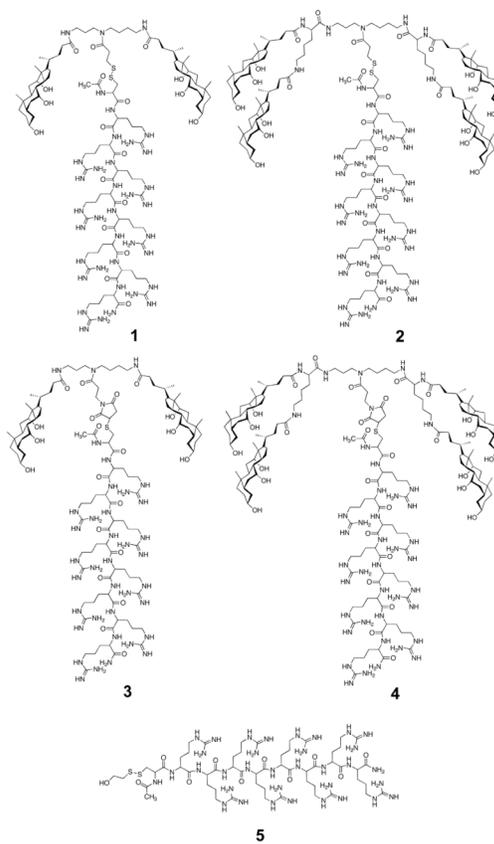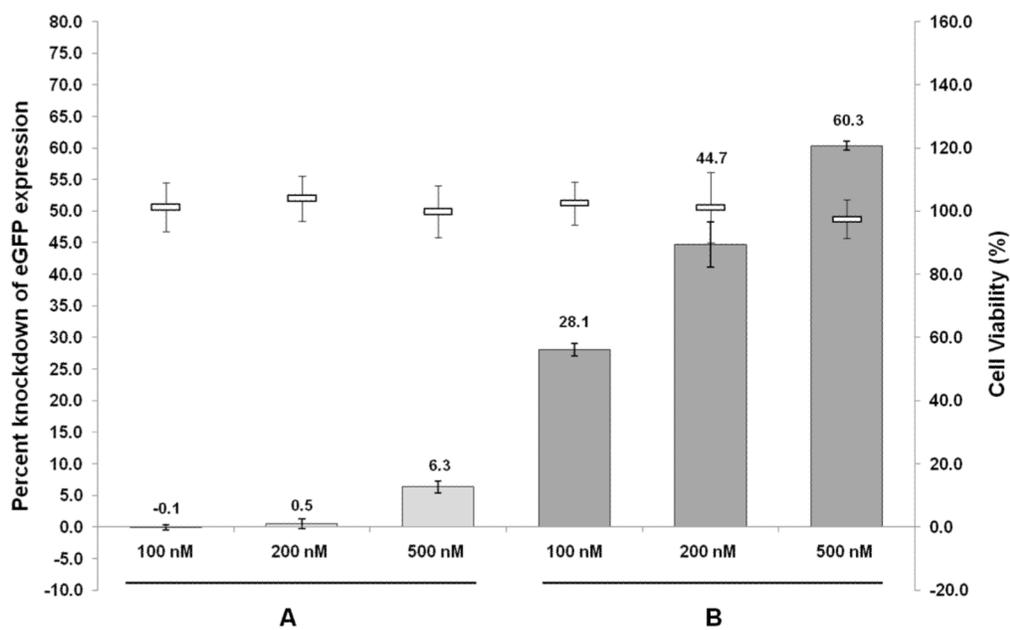
**Figure 2.**
Molecular structures of umbrella-octaarginine conjugates **1**, **2**, **3** and **4**, and control peptide, **5**.

**Figure 3.**
Percentage of eGFP knockdown (bar graph) and cell viability (open rectangles) using varying concentrations of **1** (DW-S-S-**Pep**), **2** (TW-S-S-**Pep**), and **5** (OH-S-S-**Pep**) as compared to cells which were untreated. Lipofectamine 2000 was used as described by the manufacturer. Values for percent knockdown of eGFP expression are given above each column, and represent the average of a typical experiment done in triplicate. Error bars represent the standard deviation of these values.

**Figure 4.**
Percentage of eGFP knockdown (bar graph) and cell viability (open rectangles) using varying concentrations of **3** (DW-mal-**Pep**) and **4** (TW-mal-**Pep**) as compared to cells which are untreated. Lipofectamine 2000 was used as described by the manufacturer. Values for percent knockdown of eGFP expression are given above each column, and represent the average of a typical experiment done in triplicate. Error bars represent the standard deviation of these values

**Figure 5.**
Percentage of eGFP knockdown (bar graph) and cell viability (open rectangles) for **4** when the molecular umbrella-octaarginine conjugate was (A) first incubated with the HeLa cells prior to addition of the eGFP siRNA, or (B) pre-mixed with the eGFP siRNA, followed by incubation with the HeLa cells. Values for percent knockdown of eGFP expression are given above each column, and represent the average of a typical experiment done in triplicate. Error bars represent the standard deviation of these values
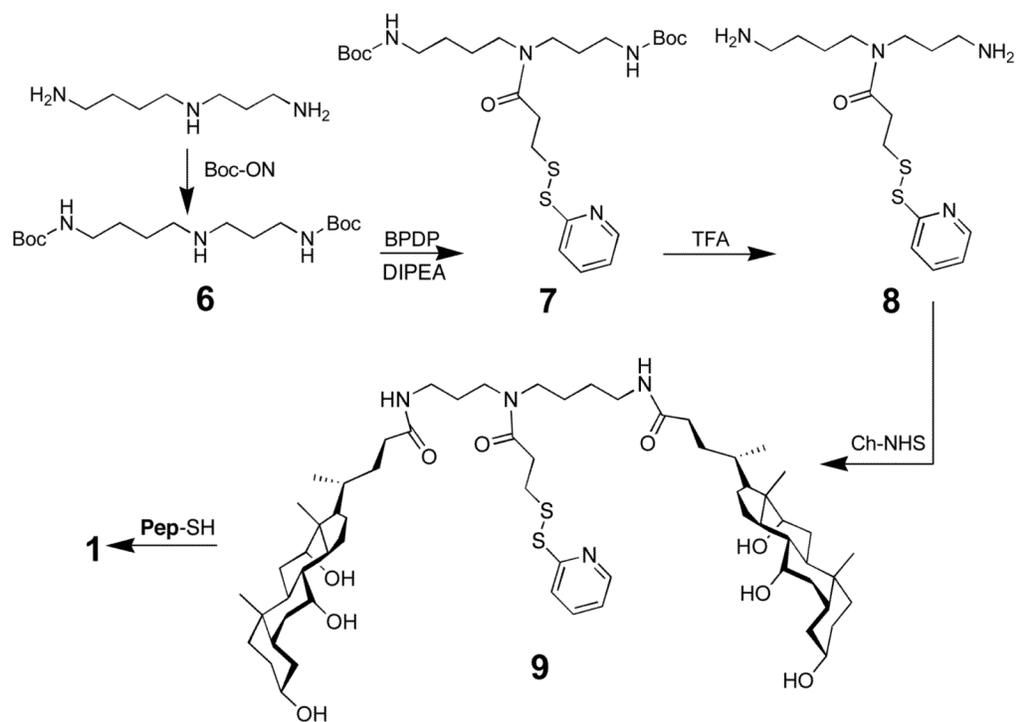
**Scheme 1.**
Synthetic approach used for the synthesis of 1
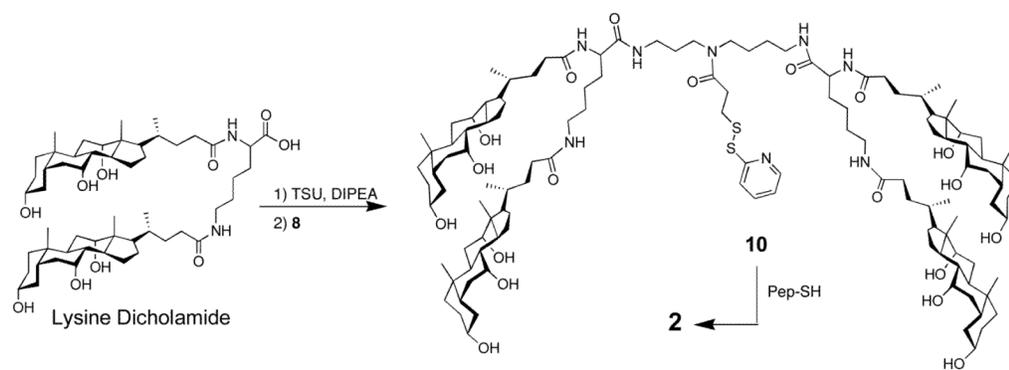
**Scheme 2.**
Synthetic approach used for the synthesis of 2
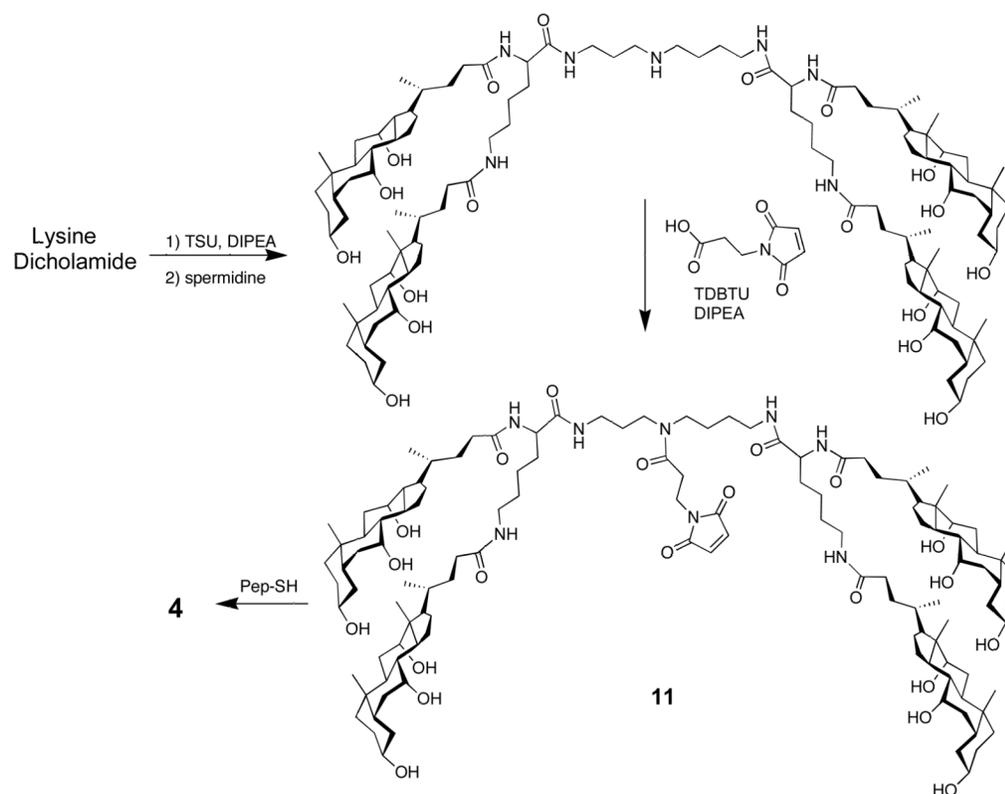
**Scheme 3.**
Synthetic approach used for the synthesis of 4