ORGANIC LETTERS

2005
Vol. 7, No. 22
4815−4818

Dendrimeric Molecular Transporters: Synthesis and Evaluation of Tunable Polyguanidino Dendrimers That Facilitate Cellular Uptake

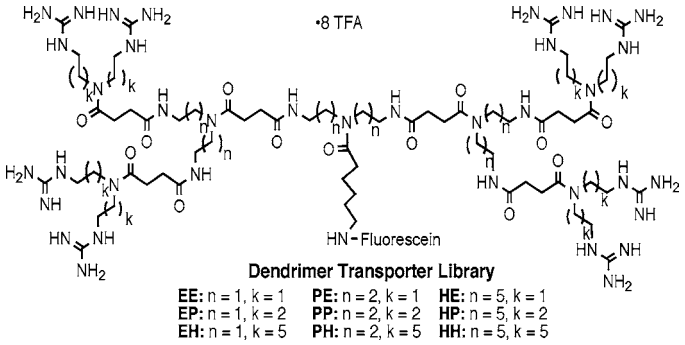
Paul A. Wender,*,† Erik Kreider,‡ Erin T. Pelkey,† Jonathan Rothbard,* and Christopher L. VanDeusen*,†

Department of Chemistry and Department of Molecular Pharmacology, Stanford University, Stanford, California 94305-5080, and Cellgate, Inc., 552 Del Rey Avenue, Sunnyvale, California 94086

wenderp@stanford.edu

Received June 27, 2005 *(Revised Manuscript Received September 14, 2005)*

ABSTRACT



Nine fluorescently labeled structurally varied polyguanidino dendrimers based on diamino acid monomeric units were individually synthesized in an efficient, scalable sequence using a trifluoroacetamide protecting group–perguanidinylation strategy. While the dendrimers varied significantly in their ability to enter a human lymphocyte cell line, the best transporters out-performed an oligoarginine reference standard.

The identification of molecular transporters that enable or enhance cellular uptake of drug candidates and probes that cannot enter cells or do so only poorly is a challenge of great significance in chemistry, biology, and medicine.[1,2] HIV-Tat-derived molecules, most notably Tat_{49-57} (RKKRRQRRR), have proven to be important leads in this area, serving as transporters for the uptake of a wide range of cargos.[2] Structure−function studies from our laboratories (P.A.W., J.R.) have shown that oligomers of L-arginine outperform Tat_{49-57} in many uptake assays.[2a] While the number of arginines in an oligomer influences uptake up to a point, 8-mers (RRRRRRRR) offer the best match of performance and cost of goods. D-Oligomers of arginine work as well or better than L-isomers due presumably to protease resistance. The first peptoids corresponding to the arginine oligomers (e.g., N-arg9) were also synthesized and found to exhibit superior uptake into cells.[3] We subsequently showed that interdigitation of the arginine backbone with other α,

† Stanford University.
‡ Cellgate, Inc.

(1) Snyder, E. L.; Dowdy, S. F. *Expert Opin. Drug Del* **2005**, *2*, 43−51.

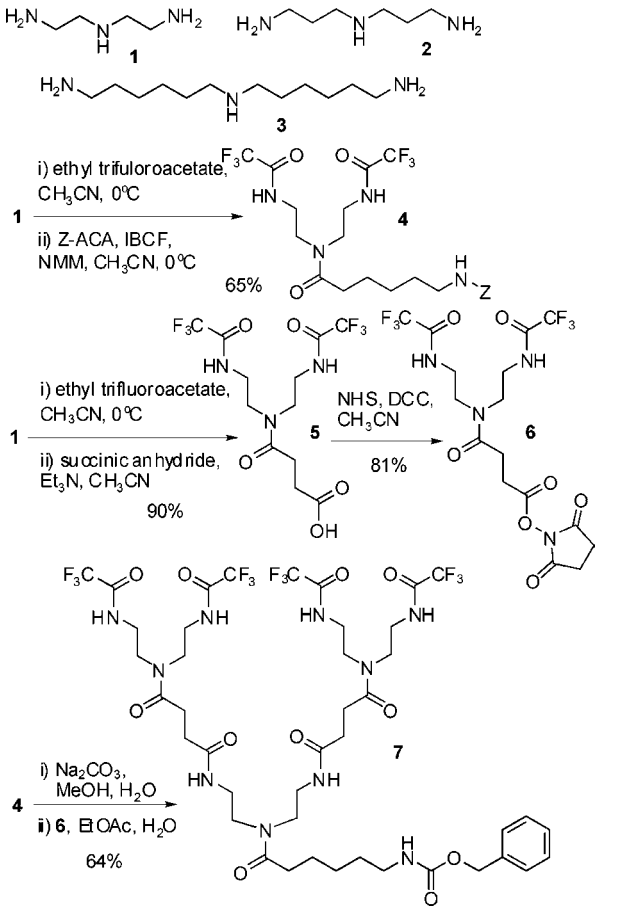
(2) (a) Rothbard, Jonathan B.; Kreider, Erik; Pattabiraman, Kanaka; Pelkey, Erin T.; VanDeusen, Christopher L.; Wright, Lee; Wylie, Bryan L.; Wender, Paul A. In *Cell-Penetrating Peptides: Processes and Applications*; Langel, Ü., Ed.; CRC Press: Boca Raton, FL, 2202; pp 141−160. (b) Frankel, A. D.; Pabo, C. O. *Cell* **1988**, *55*, 1189−1193. (c) Langel, Ü. *Cell-Penetrating Peptides*; CRC Press LLC: Boca Raton, FL, 2002. (d) Futaki, S.; Suzuki, T.; Ohashi, W.; Yagami, T.; Tanaka, S.; Ueda, K.; Sugiura, Y. *J. Biol. Chem.* **2001**, *276*, 5836−5840.

(3) Wender, P. A.; Mitchell, D. J.; Pattabiraman, K.; Pelkey, E. T.; Steinman, L.; Rothbard, J. B. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13003−13008.

10.1021/ol051496y CCC: $30.25
Published on Web 09/27/2005

β, γ, and δ amino acids (RXRXRXRXRXRXRXR)[4] or even replacement of the peptidic backbone with an oligocarbamate improved cellular uptake relative to the corresponding arginine oligomers.[5] Collectively, these results suggest there is not a single optimal position for arginine along the linear chain, but rather that the number of guanidinium headgroups in a transporter is the principal determinant of cellular uptake. An adaptive translocation mechanism accommodating these and other observations has recently been proposed.[6] Of special note, we found that these arginine oligomers with attached drugs also penetrate tissue including human skin, enabling their entry into human clinical trials.[3,7] Concurrent with these studies in which guanidinium groups are attached to a *linear* backbone, we started several years ago to investigate the synthesis and performance of transporters based on a dendrimeric backbone to examine the effect of branching on cellular uptake.[8] Our initial studies of these new transporters are reported herein.

Scheme 1. Synthesis of Tetrameric Dendrimers



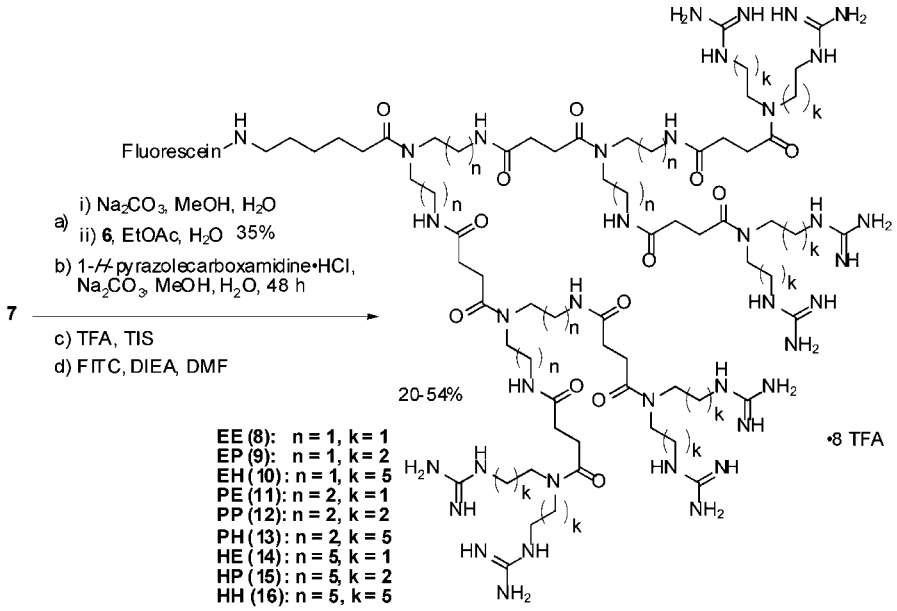
Dendrimers represent an attractive transporter scaffold that offers the advantage of step economical assembly of octaguanidinium transporters through a variant (Schemes 1 and 2) of a segment-doubling strategy (2 → 4 → 8) that we previously reported for the synthesis of octaarginine transporters.[9] Specifically, each arm of a two-arm dendrimer core could be extended with a two-arm segment to give a four-arm first generation product. Repetition of this cycle would then lead to an eight-arm system capable of incorporating eight guanidinium groups, the number that has worked well for cellular uptake using a linear backbone.[2a] Typically, dendrimers branch out symmetrically from a central point (i.e., PAMAM).[10] This architecture precludes covalent attachment of a drug or probe. However this problem could be circumvented through the use of "arboreal" dendrimers based a triamine building block.[11] An alternative architecture based on amino triol subunits has been independently reported by Goodman and co-workers.[12] Arboreal dendrimers (named for their tree-like structure) possess an orthogonal core in which the dendrimer branches out from one end.[11] This results in a structure whose branches could accommodate guanidinium groups while a cargo could be attached to its trunk.[11] One of the more exciting arboreal dendrimers to come out in the past few years in terms of ease of synthesis are the triamine based dendrimers. These dendrimers consist simply of a triamine core that has been used to open an acid anhydride to generate a diamino acid.[13] These monomers are analogous to lysine but lack the stereochemistry and are synthetically much more readily varied.[13c] Recently, they have been used as templates for the assembly of fluorescent probes as well as saccharides.[13a,c,14] Herein, we report the use of subunit tunable, triamine based diamino acids to readily form polyguanidinylated dendrimers that rapidly enter cells.

Theoretically, there are two ways to assemble an arboreal dendrimer. In the first approach, the terminal generation is assembled first and attached to cores with fewer and fewer sites of functionality in a convergent synthesis. In the second approach, the dendrimer is synthesized from an orthogonal core and symmetrical branches attached with each successive generation containing a greater number of terminal functional groups in a divergent synthesis. We chose the divergent approach to provide an orthogonally derivatized core.

Shown in Schemes 1 and 2 for clarity is the representative

(4) Rothbard, J. B.; Kreider, E.; VanDeusen, C. L.; Wright, L.; Wylie, B. L.; Wender, P. A. *J. Med. Chem.* **2002**, *45*, 3612−3618.
(5) Wender, P. A.; Rothbard, J. B.; Jessop, T. C.; Kreider, E. L.; Wylie, B. L. *J. Am. Chem. Soc.* **2002**, *124*, 13382−13383.
(6) (a) Rothbard, J. B.; Jessop, T. C.; Lewis, R. S.; Murray, B. A.; Wender, P. A.*J. Am. Chem. Soc.* **2004**, *126*, 9506−9507. (b) Rothbard, J. B.; Jessop, T. C.; Wender, P. A. *Adv. Drug Del. Rev.* **2005**, *57*, 495−504.
(7) (a) Rothbard, J. B.; Garlington, S.; Lin, Q.; Kirschberg, T.; Kreider, E.; McGrane, P. A.; Wender, P. A.; Khavari, P. A. *Nat. Med.* **2000**, *6*, 1253−1257.
(8) VanDeusen, C. L. Ph.D. Thesis, Guanidinium-Rich Molecular Transporters for Drug Delivery, Department of Chemistry, Stanford University, 2003.
(9) Wender, P. A.; Jessop, T. C.; Pattabiraman, K.; Pelkey, E. T.; VanDeusen, C. L.*Org. Lett.* **2001**, *3*, 3229−3232.
(10) Tomalia, D. A.; Naylor, A. M.; Goddard, W. A., III. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 138−175.
(11) Newkome, G. R.; Yao, Z. Q.; Baker, G. R.; Gupta, V. K. *J. Org. Chem.* **1985**, *50*, 2003−2004.
(12) (a) Chung, H. H.; Harms, G.; Min Seong, C.; Choi, B. H.; Min, C.; Taulane, J. P.; Goodman, M. *Biopolymers* **2004**, *76*, 83−96. (b) Goodman, M.; Seong, C. M.; Harms, G.; Min, C.; Choi, B. H.; Chung, H. H. Patent WO 2004009666.
(13) (a) Adamczyk, M.; Fishpaugh, J.; Heuser, K. *Org. Prep. Proced. Intl.* **1998**, *30*, 339−348. (b) Adamczyk, M.; Fishpaugh, J.; Mattingly, P. G.; Shreder, K. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 3595−3598. (c) Llinares, M.; Roy, R. *Chem. Commun.* **1997**, 2119−2120.
(14) (a) Veprek, P.; Jezek, J. *J. Pept. Sci.* **1999**, *5*, 5−23. (b) Veprek, P.; Jezek, J. *J. Pept. Sci.* **1999**, *5*, 203−220.

Scheme 2. Synthess of Octamer Dendrimer and Perdeprotection/Perguanidinylation



synthesis of the dendrimers from triamine **1**. The exact same sequence of monomer synthesis and assembly was repeated for each of the triamines **1**−**3** to produce the family of nine dendrimers shown in Scheme 2. The synthesis of the core began with the selective protection of the primary amines in **1** with ethyl trifluoroacetate (Scheme 1). Solvent removal followed by reaction of the crude amine with the isobutyl mixed carbonic anhydride of *Z*-aminocaproic acid yielded the core element **4** that was ready for expansion (Scheme 1).

The synthesis of the acid proceeded with the same primary trifluoracetamide protection as above but was followed by reaction of the crude product with succinic anhydride. Extractive workup yielded the diamine-protected acid **5** (Scheme 1). Attempts to activate this acid in situ (i.e., acid chloride, DCC, isobutyl chloroformate) to react with the diamine core during the expansion of the dendrimer led to complex mixtures of products. Therefore, the *N*-hydroxysuccinimidyl ester **6** of the acid **5** was synthesized and was found to provide clean acylation reactions in subsequent steps (Scheme 1).

The subsequent acylation procedure that was ultimately developed involved first removing the primary trifluoroacetamides of **4** with sodium carbonate in aqueous methanol. Following removal of the methanol in vacuo, the crude polyamine solution was reacted using a *biphasic* acylation for which the *N*-hydroxysuccinimidyl ester **6** was introduced in ethyl acetate. After separation of the organic and aqueous phases, the product **7**, along with unreacted ester can be obtained by flash chromatography (Scheme 1). This procedure proved to be the most effective, as the isolation of the polyamines was difficult. To complete the synthesis after the second expansion of the core (generation three, Scheme 2), the various octatrifluoroacetamides were perguanidinylated using the perdeprotection/perguanidinylation procedure our group reported for the synthesis of arginine oligomers from the corresponding ornithines.[9] Removal of the benzyl carbamate using trifluoroacetic acid yielded the dendrimer transporters in a total of 8 steps following purification by preparative HPLC. This is comparable in step economy to our previously reported segment doubling strategy for octaarginine synthesis.[9]

To study the effect of spacer length in the dendrimer on cell uptake activity, nine dendrimers **EE-HH** (Scheme 2, **8**−**16**) were synthesized with varying numbers of methylenes (diethylene triamine, 3-aminopropylpropane diamine, and hexamethylene triamine). To study the effect of the terminal dendrimer generation on activity, each triamine **1**−**3** (Scheme 1) was used to synthesize the first two generations of dendrimer. At this stage, each of these core dendrimers was capped with each of the three corresponding diamino acids, allowing overall for the parallel synthesis of nine dendrimers, differing by the spacing between their cores and their terminal guanidino groups (Scheme 2).

Varying concentrations of the fluorescently labeled dendrimers, **EE-HH** in Scheme 2 were dissolved in phosphate buffered saline pH 7.4 (PBS) and incubated with 3×10^5 transformed human T lymphocytes, Jurkat, for 3 min. Following this, the fluorescent dendrimer was removed from the cells by centrifugation, and the resultant cell pellet was washed with PBS containing 2% fetal calf serum, spun, and resuspended in PBS containing 2% fetal calf serum. The fluorescence in the resulting cell suspension was analyzed by flow cytometry (Figure 1).

Even though all dedrimeric transporters had an identical number of guanidinium groups, they exhibit a wide range of uptake into lymphocytes. Several patterns are apparent, with the most obvious being that the longer and more flexible the dendrimeric hydrocarbon chains, the better the uptake. The hexyl hexyl (**HH**; **16**) and the hexyl propyl (**HP**; **15**)

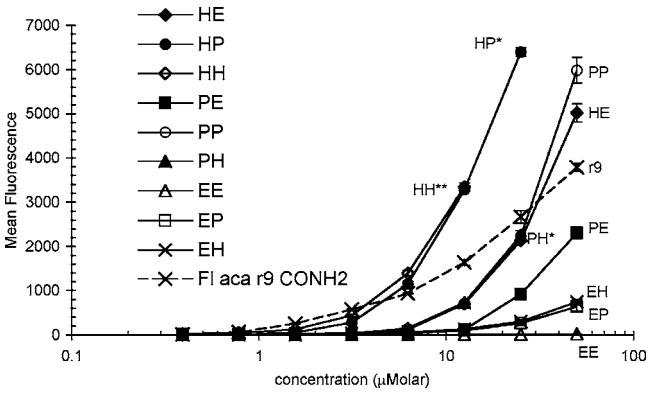


Figure 1. Comparison of the mean fluorescence as measured by flow cytometry of the nine dendrimers (**8**−**16**) and a fluorescenated nonamer of D-arginine (**r9**: a linear transporter) after a 3-min incubation with the human T cell line Jurkat. Each point is the average fluorescence of 10000 live cells (propidium iodide negative), measured in triplicate. In all but three cases, the viability of the cells was greater than 95%. Data were not included for HH** at 25 and 50 μM and at 50 μM for HP* and PH* due to greater than 20% cell death.

dendrimers entered the cells most effectively, while the ethyl ethyl analogue (**EE**; **8**) could not be detected within the cells during the time course of the experiment. The nine compounds can be placed into five groups, the most effective, (**HH** and **HP**), the second fastest group, (**HE**, **PP**, **PH**), marginal transporters (**EH** and **EP**), a nontransporter (**EE**), and an intermediate transporter (**PE**), less effective than the second group, but better than the marginal transporters.

These cellular uptake results show some important correlations with our earlier work on peptoid analogues of the arginine based molecular transporters, in which peptoids with longer hydrocarbon side chains and greater flexibility exhibited faster rates of uptake into cells.[3] If the dendrimers are divided into families with identical cores, the dendrimers with hexyl or propyl spacing at the terminal generation in all cases are more effective than the ethyl spaced transporters in entering cells, i.e., **EH**,**EP** > **EE**; **PH**,**PP** > **PE**; **HH**,**HP** > **HE**. If the core spacing is compared, the same trends hold true, with longer, more lipophillic dendrimers displaying faster uptake, in the order **HH**,**HP** > **PP**,**PH** > **EH**,**EP** and **HE** > **PE** ≫ **EE**. A final point that might have mechanistic implications is that the curve shapes of the more effective dendrimers are quite different from that of a fluoresceinated conjugate of nonaarginine (r9). The more effective dendrimers outperform the nonaarginine conjugate at higher concentrations (Figure 1). While interpretation of these observations requires further investigation, these results are consistent with an adaptive translocation mechanism[6a] involving transient association of the guanidinium headgroups with cell surface groups (e.g., phosphates, carboxylates, sulfates), which serves to enable passage through the membrane as a transient lipidated ion pair complex.

Given the effective performance of several of these dendrimers and their expected use for cargo delivery, we investigated whether the synthesis described above could be conducted on a larger scale (grams). The synthesis proved robust providing 14.5 g of the fully protected all propyl octamer (**PP**) with no procedural modifications. A 1.5 g portion of this supply was then perguanadinylated to yield over a gram of the final product (**PP**: **12**).

In summary, we report the synthesis and evaluation of dendrimeric molecular transporters. The transporters were synthesized in eight steps from inexpensive triamines **1**−**3** using biphasic acylations to expand the dendridic core. Fluorescently labeled dendrimers were evaluated for their cellular uptake kinetics. The longer, more lipophilic, and/or more flexible spacing in the dendrimers resulted in the most effective transport into cells exceeding that of even oligoarginine transporters at high concentrations.

Acknowledgment. Support of this work by grants from the National Institutes of Health (CA31841, CA31845) is gratefully acknowledged.

Supporting Information Available: Experimental results and procedures for the synthesis and assays. This material is available free of charge via the Internet at http://pubs.acs.org.

OL051496Y