[14] a) Y. Oikawa, K. Sugano, O. Yonemitsu, *J. Org. Chem.* **1978**, *43*, 2087 – 2088; b) Y. Oikawa, T. Yoshioka, K. Sugano, O. Yonemitsu, *Org. Synth.* **1985**, *63*, 198 – 202; c) Y. Yamamoto, Y. Watanabe, S. Ohnishi, *Chem. Pharm. Bull.* **1987**, *35*, 1860 – 1870; d) Y. Oikawa, T. Yoshioka, K. Sugano, O. Yonemitsu, *Org. Synth. Collected Vol. 7*, 359 – 361.
[15] I. H. Gilbert, M. Ginty, J. A. O'Neill, T. J. Simpson, J. Staunton, C. L. Willis, *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1587 – 1590.
[16] C. E. Anson, M. J. Bibb, K. I. Booker-Milburn, C. Clissold, P. J. Haley, D. A. Hopwood, K. Ichinose, W. P. Revill, G. R. Stephenson, C. M. Surti, *Angew. Chem.* **2000**, *112*, 230 – 233; *Angew. Chem. Int. Ed.* **2000**, *39*, 224 – 227.
[17] A. Jacobs, J. Staunton, A. Sutkowski, *J. Chem. Soc. Chem. Commun.* **1991**, 1113 – 1114.
[18] J. Crosby, D. H. Sherman, M. J. Bibb, W. P. Revill, D. A. Hopwood, T. J. Simpson, *Biochim. Biophys. Acta* **1995**, *1251*, 32 – 42.
[19] Covalent disulfides form rapidly in the absence of dithiothreitol (DTT), linking through the terminal PP thiol to either the thiol of C17 or the PP thiol of another holo-ACP. In these forms, ACP is catalytically inactive. The use of a C17S mutant and preincubation with DTT avoids these pitfalls. See ref. [12] for more details.
[20] W. P. Revill, M. J. Bibb, D. A. Hopwood, *J. Bacteriol.* **1995**, *177*, 3946 – 3952.
[21] At 5mM **5b** more acyl ACP and ACP SNAC was evident, but addition of MCAT made no detectable difference.
[22] W.Humphrey, A. Dalke, K. Schulten, *J. Mol. Graphics* **1996**, *14*, 33 – 38. See http://www.ks.uiuc.edu/Research/vmd/.
[23] N. Guex, M. C. Peitsch, *Electrophoresis*, **1997**, *18*, 2714 – 2723. See http://www.expasy.ch/spdbv/.

Received: August 23, 2001 [Z 290]

Cellular Uptake Studies with β-Peptides

Magnus Rueping,[a] Yogesh Mahajan,[a] Markus Sauer,*[b] and Dieter Seebach*[a]

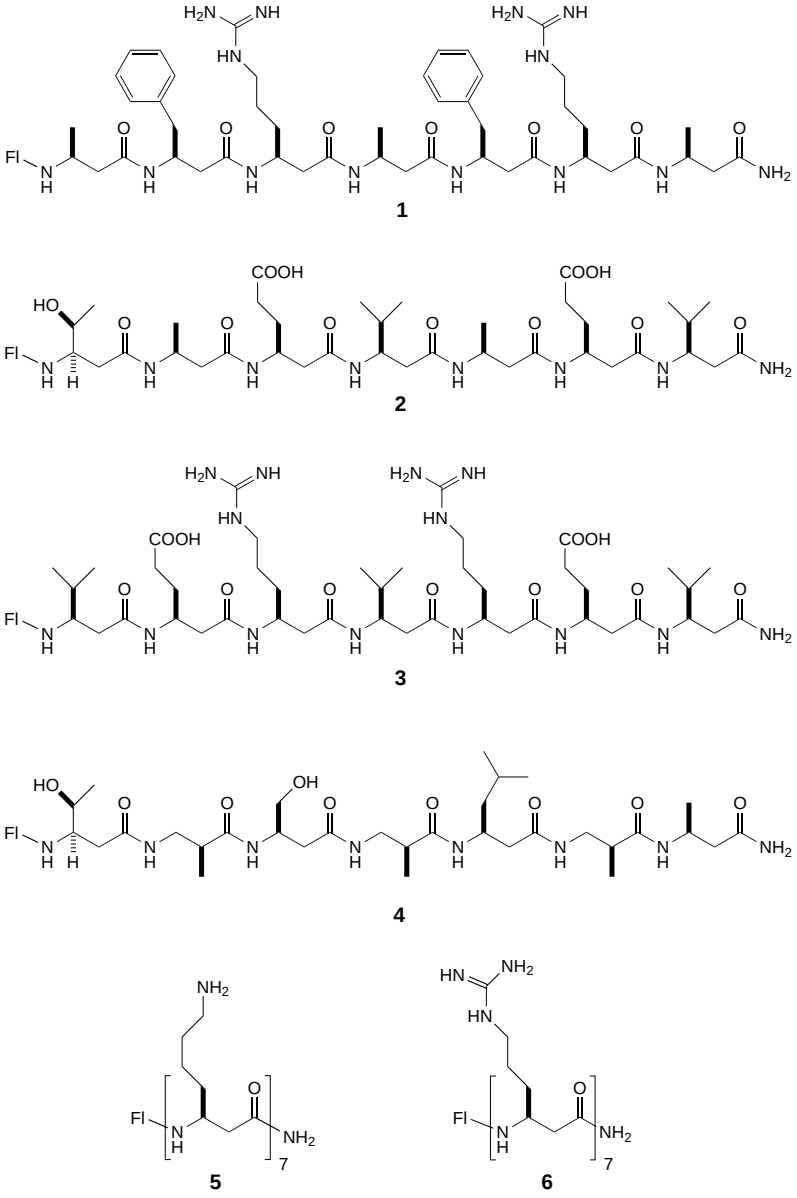
KEYWORDS:

arginine · cellular uptake · fluorescence microscopy · peptides

β-Peptides have been shown to fold into stable secondary structures similar to those observed in natural peptides and proteins.[1] The finding that β-peptides are completely stable to proteolytic degradation renders them candidates for use as peptidomimetics.[2] In fact, β-peptides have been demonstrated to mimic α-peptidic hormones,[3] to inhibit cholesterol uptake,[4] and to possess antimicrobial[5] and antiproliferative properties.[6]

The bioavailability of pharmacologically active compounds, for instance, peptides and proteins, depends significantly on their physical properties, as their uptake correlates with solubility in the polar extracellular medium and passive diffusion through the nonpolar lipid bilayer. Recently, methods have been developed for the delivery of proteins, biological active compounds, and DNA into living cells with the help of membrane-permeable carrier peptides (oligomers containing basic side chains).[7]

In order to determine the structural requirements for cellular uptake of β-peptides, a series of fluorescein-labeled β-peptides **1** – **6** has been prepared (Scheme 1). The substitution pattern of the β-peptides was selected for the following reasons: the β-



Scheme 1. *Molecular formulas of the investigated fluorescein-labeled β-peptides* ***1*** *–* ***6****. Fl = fluorescein.*

[a] *Prof. Dr. D. Seebach, M. Rueping, Y. Mahajan*
Laboratorium für Organische Chemie
der Eidgenössischen Technischen Hochschule
ETH-Hönggerberg
8093 Zürich (Switzerland)
Fax: (+41) 1-632-11-44
E-mail: seebach@org.chem.ethz.ch

[b] *Dr. M. Sauer*
Physikalisch-Chemisches Institut
Universität Heidelberg
Im Neuenheimer Feld 253
69120 Heidelberg (Germany)
E-mail: Sauer@urz.uni-heidelberg.de

peptides **1** – **3** should be able to form a 3_{14} helix[8] (**1** has two positively charged, **2** has two negatively charged, and **3** has both positively and negatively charged side chains). While the β-peptide **3** has been designed to form a saltbridge-stabilized 3_{14} helix,[9] the β-heptalysine **5** and the β-heptaarginine **6** are not expected to form a helix because of the destabilizing effects of adjacent cationic side chains.[10] β-Peptide **4** consists of alternating β^2- and β^3-amino acids, a substitution pattern that has been shown to induce a nonpolar 10/12-helical structure.[11] The β-peptides **1** – **6** were synthesized by standard solid-phase methods and fluorescein-labeled at the N terminus prior to cleavage from the Rink amide resin.

The ability of the fluorescence-labeled peptides to enter the cells was analyzed by fluorescence microscopy. 3T3 mouse fibroblast cells were cultured as exponentially growing monolayers in RPMI 1640 medium, without phenol red, supplemented with 10% (v/v) fetal calf serum (FCS) and 1 mM glutamine, at 37 °C under 5% CO_2. Fluorescence-labeled peptides (up to a final concentration of 1 μM) were added to fibroblast cells grown on chambered cover glasses in FCS medium and incubated for 10 – 40 min at 37 °C. Subsequently, cells were rinsed twice with phosphate-buffered saline (pH 7.3) at room temperature and suspended in FCS medium for measurements. All live-cell measurements were done at 20 °C by using a laser scanning microscope with a 63 × oil immersion objective. The internalization of free fluorescein under the same conditions was tested simultaneously.

For β-peptides **1** – **4**, as well as for the free fluorescein, no uptake into the cells was observed with the described experimental conditions in any of the experiments. However β-peptides **5** and **6**, the polycationic β-oligolysine and β-oligoarginine, were translocated through the membrane, with the arginine derivative **6** internalizing to a much greater extent. After treatment with fluorescein-labeled β-heptaarginine **6** (1 μM) for 40 min accumulation was observed in the cytosol and nucleus, with concentration in the nucleoli (Figure 1).

No toxicity was observed, even with longer incubation times (12 h), at 1 μM concentrations at 37 °C in 10% (v/v) FCS medium.[12] Interestingly, some compartments in the cytoplasm showed lower fluorescence intensities. Most probably these compartments represent mitochondria where the fluorescein moieties are protonated due to the lower pH value and, therefore, exhibit only low fluorescence quantum yields.

When the cells were incubated with β-heptaarginine **6** at 20 °C and analyzed after just 2 min without washing, weak fluorescence is detected (Figure 2 a); after 5 min the fluorescence is almost as intensive as after 40 min (Figure 2 b). These data suggest that saturation is already occurring a few minutes after treatment with 1 μM β-heptaarginine solutions. Incubation of cells with **6** for 10 min in the temperature range from 4 – 37 °C exhibited comparable translocation activity. Consistent with previous measurements on arginine-rich peptides,[7] these data indicate that cell penetration by β-heptaarginine does not depend on endocytosis. The experiments described prove the ability of polycationic β-peptides to internalize into cells. β-oligoarginine **6** was significantly more effective in entering cells than β-oligolysine **5**.

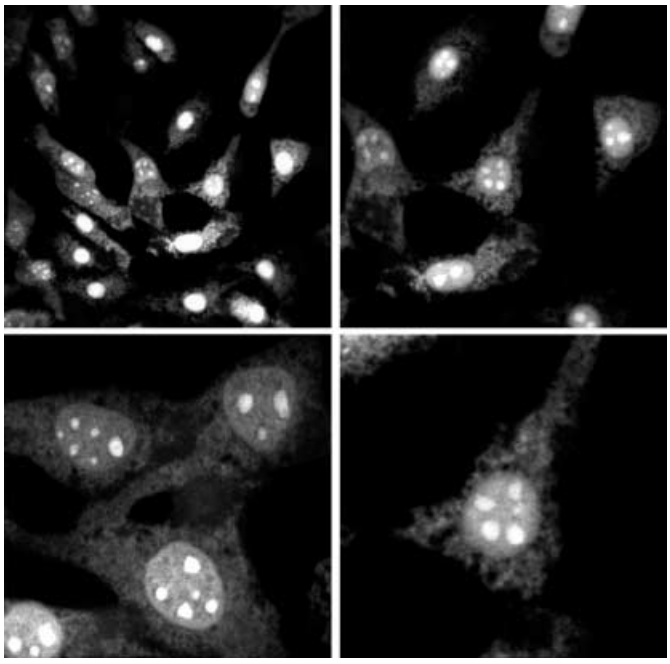


Figure 1. *Fluorescence microscopy investigation of cells treated with fluorescein-labeled β-oligoarginine **6** (1 μM) for 40 min. After incubation the cells were rinsed twice. Accumulation of **6** is observed in the cytosol and nucleus, with nucleolar concentrations.*

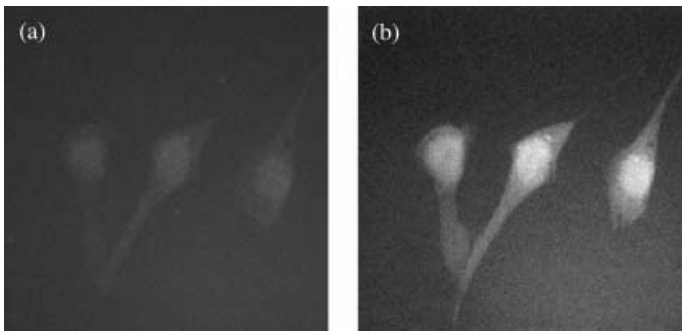


Figure 2. *Time-dependent uptake measurements of β-oligoarginine **6** (1 μM, no washing) at 20 °C already show a little internalization after 2 minutes of incubation (a); the cells show much intense fluorescence after 5 minutes (b).*

Due to their resistance towards enzymatic degradation[2, 13] β-oligoarginine derivatives might be used for long-term binding to cell nuclei; this technique could lead to applications for diagnostic purposes or for transport of antisense DNA-binding structures. Also, β-oligoarginine could be used to internalize otherwise nonpenetrating compounds (peptides, proteins) into cells. The higher stability of β-oligoarginine, as compared to the α-peptidic analogues,[14] might be useful for elucidating the mechanism by which oligoarginines pass the cell walls.[15]

[1] a) D. Seebach, J. L. Matthews, *Chem. Commun.* **1997**, 2015 – 2022; b) S. H. Gellman, *Acc. Chem. Res.* **1998**, *31*, 173 – 180; c) K. Gademann, T. Hintermann, J. V. Schreiber, *Curr. Med. Chem.* **1999**, *6*, 905 – 925; d) R. P. Cheng, S. H. Gellman, W. F. DeGrado, *Chem. Rev.* **2001**, *101*, 3219 – 3232; e) D. J. Hill, M. J. Mio, R. B. Prince, T. S. Hughes, J. S. Moore, *Chem. Rev.* **2001**, *101*, 3893 – 4011.

  1439-4227/02/03/02-03 $ 17.50+.50/0 ChemBioChem **2002**, No. 02-03

[2] a) D. Seebach, M. Overhand, F. N. M. Kühnle, B. Martinoni, L. Oberer, U. Hommel, H. Widmer, *Helv. Chim. Acta* **1996**, *79*, 913 – 941; b) T. Hintermann, D. Seebach, *Chimia* **1997**, *51*, 244 – 247; c) D. Seebach, S. Abele, J. V. Schreiber, B. Martinoni, A. K. Nussbaum, H. Schild, H. Schulz, H. Hennecke, R. Woessner, F. Bitsch, *Chimia* **1998**, *52*, 734 – 739; d) J. Frackenpohl, P. I. Arvidsson, J. V. Schreiber, D. Seebach, *ChemBioChem* **2001**, *2*, 445 – 455.

[3] a) K. Gademann, M. Ernst, D. Hoyer, D. Seebach, *Angew. Chem.* **1999**, *111*, 1302—1304; *Angew. Chem. Int. Ed.* **1999**, *38*, 1223 – 1226; b) K. Gademann, T. Kimmerlin, D. Hoyer, D. Seebach, *J. Med. Chem.* **2001**, *44*, 2460 – 2468.

[4] M. Werder, H. Hauser, S. Abele, D. Seebach, *Helv. Chim. Acta* **1999**, *82*, 1774 – 1783.

[5] a) Y. Hamuro, J. P. Schneider, W. F. DeGrado, *J. Am. Chem. Soc.* **1999**, *121*, 12 200 – 12 201; b) E. A. Porter, X. Wang, H. S. Lee, B. Weisblum, S. H. Gellman, *Nature* **2000**, *405*, 298 – 298; c) P. I. Arvidsson, J. Frackenpohl, N. S. Ryder, B. Liechty, F. Petersen, H. Zimmermann, G. P. Camenisch, R. Woessner, D. Seebach, *ChemBioChem* **2001**, *2*, 771 – 773; d) D. H. Liu, W. F. DeGrado, *J. Am. Chem. Soc.* **2001**, *123*, 7553 – 7559.

[6] K. Gademann, D. Seebach, *Helv. Chim. Acta* **2001**, *84*, 2924 – 2937.

[7] a) M. Lindgren, M. Hallbrink, A. Prochiantz, U. Langel, *Trends Pharmacol. Sci.* **2000**, *21*, 99 – 103; b) S. R. Schwarze, S. F. Dowdy, *Trends Pharmacol. Sci.* **2000**, *21*, 45 – 48; c) D. J. Mitchell, D. T. Kim, L. Steinman, C. G. Fathman, J. B. Rothbard, *J. Pept. Res.* **2000**, *56*, 318 – 325; d) S. Futaki, T. Suzuki, W. Ohashi, T. Yagami, S. Tanaka, K. Ueda, Y. Sugiura, *J. Biol. Chem.* **2001**, *276*, 5836 – 5840; e) P. A. Wender, D. J. Mitchell, K. Pattabiraman, E. T. Pelkey, L. Steinman, J. B. Rothbard, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 13 003 – 13 008.

[8] D. Seebach, P. E. Ciceri, M. Overhand, B. Jaun, D. Rigo, L. Oberer, U. Hommel, R. Amstutz, H. Widmer, *Helv. Chim. Acta* **1996**, *79*, 2043 – 2066.

[9] P. I. Arvidsson, M. Rueping, D. Seebach, *Chem. Commun.* **2001**, 649 – 650.

[10] S. Abele, G. Guichard, D. Seebach, *Helv. Chim. Acta* **1998**, *81*, 2141 – 2156.

[11] D. Seebach, K. Gademann, J. V. Schreiber, J. L. Matthews, T. Hintermann, B. Jaun, L. Oberer, U. Hommel, H. Widmer, *Helv. Chim. Acta* **1997**, *80*, 2033 – 2038.

[12] Cell viability was verified by using trypan blue.

[13] J. Frackenpohl, unpublished results; β-peptides containing β-oligoarginine have been tested for stability against pronase, proteinase K, chymotrypsin, trypsin, carboxypeptidase A, and 20S Proteasome; for the methodology, see ref. [2d].

[14] E. Vives, P. Brodin, B. Lebleu, *J. Biol. Chem.* **1997**, *272*, 16 010 – 16 017.

[15] **Note added in proof:** After submission of this paper we learned about a contribution by Gellman et al. (N. Umezawa, M. A. Gelman, M C. Haigis, R. T. Raines, S. H. Gellman, *J. Am. Chem. Soc.* **2002**, *124*, 368 – 369), which describes very much the same results. We first reported cellular uptake of a fluorescence-labeled β-peptide at the 17th American Peptide Symposium, San Diego, June 9 – 14, 2001 (lecture by D.S. on "Homologs of Amino Acids and Explorations into the Worlds of β- and γ-Peptides"). The translocation of oligo-β-HArg derivatives and binding to the cell nucleus was first disclosed by D.S. at a lecture presented to the Peptide, Protein and Proteonomics Group, Biozentrum Basel, on November 29, 2001. We thank Dr. C. Garcia-Echeverria of Novartis Pharma AG, Basel, for personal communication of the results of his independent investigation on cellular uptake of oligo-β-HArginines.

Received: December 22, 2001 [Z 337]