DOI: 10.1002/cmdc.200500037

Click Chemistry on Azidoproline: High-Affinity Dual Antagonist for HIV-1 Envelope Glycoprotein gp120

Hosahudya N. Gopi, Kalyan C. Tirupula, Sabine Baxter, Sandya Ajith, and Irwin M. Chaiken*[a]

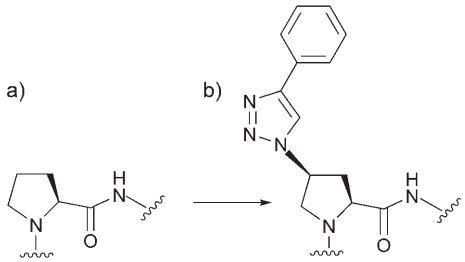
Recent advances in the Cu^{I}-catalyzed Huisgen 1,3-dipolar cycloaddition of azides and terminal alkynes afford 1,4-disubstituted 1,2,3-triazoles with superior regioselectivity, and almost quantitative transformation under extremely mild conditions.[1] The simple and robust features of this methodology have found application in drug discovery, bioconjugation, and material science.[2] Herein, we report the novel use of stable and chemically accessible azidoproline within an otherwise normally constituted solid-phase-synthesized polypeptide as a platform for side-chain bioconjugation reactions through click chemistry, and its use to introduce triazole conjugates into a dual antagonist of the HIV-1 envelope protein gp120. The procedure enables rapid generation of analogues at an internal side-chain position of the antagonist. This has led to a lead inhibitor for HIV-1 infection with affinity in the nanomolar range.

Acquired immunodeficiency syndrome (AIDS), the globally epidemic disease caused by HIV-1, has created an urgent need for new classes of antiviral agents.[3] Viral infection is initiated by the binding of gp120 of HIV-1 to the CD4 antigen on the host T cell surface.[4] The envelope glycoprotein of HIV-1 is a trimer that consists of three gp120 exterior envelope glycoproteins and gp41 transmembrane glycoproteins.[5] The binding of gp120 to CD4 promotes a conformational change in gp120 that increases its affinity for a second host-cell receptor, one of the chemokine receptors, CCR5 and CXCR4.[6] The interaction of gp120 with its receptors is believed to promote further conformational rearrangements in the HIV-1 envelope that drive fusion of the viral and host-cell membranes. Blockage of the interactions between gp120 and cell-surface receptors is an attractive goal for the prevention of HIV-1 infection through the inhibition of membrane fusion and viral entry. The feasibility of therapeutic efficacy with fusion inhibitors has been demonstrated recently.[7] A promising fusion inhibitor lead is a 12-residue peptide (RINNIPWSEAMM, **1**) which was discovered initially by phage library screening.[8] Peptide **1** inhibits the interaction between gp120 and both CD4 and 17b, an antibody that recognizes an epitope that overlaps the CCR5 binding site with affinity in the micromolar range.[9]

[a] *Dr. H. N. Gopi, K. C. Tirupula, S. Baxter, S. Ajith, Prof. I. M. Chaiken*
Department of Biochemistry and Molecular Biology
and A. J. Drexel Institute of Basic and Applied Protein Science
11102 New College Building, Drexel University College of Medicine
245 N. 15th Street, Philadelphia, PA, 19102 (USA)
Fax: (+1) 215-762-4452
E-mail: imc23@drexel.edu

Supporting information for this article is available on the WWW under http://www.chemmedchem.org or from the author.

Herein, we report that conjugation at proline 6 of peptide **1** through click chemistry leads to inhibitors with strikingly high affinity for the HIV-1 envelope and which maintain the dual inhibition of CD4 and 17b binding to the viral Env protein. The modification of proline with 4-phenyl-1,4-disubstituted 1,2,3-triazole, fabricated through a [3+2] cycloaddition reaction (Scheme 1) leads to a peptide which binds to gp120 with a K_D value of $\approx$12.7 nM and which inhibits the binding of gp120 to CD4 and CCR5 epitope ligands, with IC_{50} values of 22 and 29 nM, respectively. The peptide **1** conjugate peptide **5** is greater than two orders of magnitude more potent than unmodified peptide **1** and therefore provides a greatly improved starting point for the design of viral entry inhibitors.



Scheme 1. a) Native peptide with proline and b) peptide with substituted proline (2*S*,4*S*)-4-(4-phenyl-1*H*-1,2,3-triazol-1-yl)pyrrolidine-2-carboxamide.

In our study of the novel dual receptor-site entry inhibitor **1**, we were interested in replacing proline 6 with γ-amino proline (Amp). Pro 6 was chosen because of its proximity to Trp 7, the side chain of which was found to be highly sensitive to replacement.[8,9] Therefore, Pro 6 is close to a possible hot-spot for the stabilization of interactions with gp120. Peptides were synthesized by using Fmoc chemistry on 5-(4-aminomethyl-3,5-dimethoxyphenoxy)pentanoyl–poly(ethylene glycol)–polystyrene (PAL-PEG-PS) resin. Fmoc–*cis*-4-azidoproline was synthesized starting with the commercially available methyl ester of *tert*-butoxycarbonyl-4-hydroxyproline (Boc-Hyp-OMe). The *trans*-4-hydroxy group was converted into *trans*-4-mesylate by treatment with methanesulfonyl chloride in the presence of triethylamine in dichloromethane, followed by azide displacement with NaN_3 in *N*,*N*-dimethylformamide at 70 °C. The *cis*-4-azido group on proline was converted into *cis*-4-amine on resin by using a mixture of trimethylphosphine, dioxane, and water.[10]

A surface plasmon resonance (SPR) optical biosensor (Biacore 3000) was used to characterize the direct interactions of peptides with gp120 from the YU2 strain of HIV-1. In the direct binding experiments, YU2 gp120 was covalently immobilized (5000 resonance units (RU)) on a biosensor chip (CM5, Biacore). Real-time interactions were monitored by injecting various concentrations of peptide analytes in phosphate-buffered saline (PBS) at a flow rate of 5 $\mu L\,min^{-1}$ with association and dissociation phases of 5 min each. The monoclonal antibody 2B6R Fab fragment (raised against the interleukin-5 receptor α) was used as a control surface. Prior to analysis, the binding data were corrected for nonspecific interactions and buffer ef-

fects by subtracting the signals gathered from the reference surface and buffer injections, respectively. The direct binding analysis showed that peptide **4** (with Amp) had no appreciable affinity for gp120 (Table 1). However, the intermediate peptides

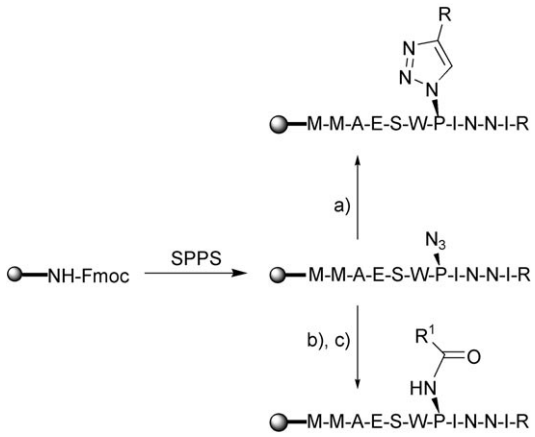
Table 1. Peptide sequences and equilibrium constants.[a]

Sequence	Peptide	K_D [μM]
RINNI(P)WSEAMM	**1**	5.16
RINNI(Hyp)WSEAMM	**2**	24.8
RINNI(Azp)WSEAMM	**3**	2.85
RINNI(Amp)WSEAMM	**4**	–

[a] Determined by direct interaction with surface-immobilized YU2 gp120 in SPR.

2 and **3**, with *trans*-4-hydroxyproline (Hyp) and *cis*-4-azidoproline (Azp), respectively, retain gp120 binding properties. Peptide **3** showed a marginally increased equilibrium constant for YU2 gp120 ($K_D = 2.85$ μM) relative to peptide **1.** The K_D values for peptides **1**–**4**, determined from direct binding analysis with immobilized gp120, are given in Table 1. Peptide **3** formed the starting point for [3+2] cycloadditions at residue 6.

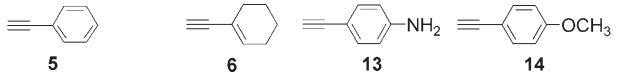
The [3+2] cycloaddition reaction of peptidyl azidoproline and terminal alkynes was carried out with an on-resin method (Scheme 2). The resin was suspended in a mixture of acetonitrile, water, DIEA, and pyridine (4:4:2:1). The R alkyne was added followed by a catalytic amount of CuI. After stirring overnight, the resin was washed with HCl (5%), DMF and dichloromethane. The peptide was cleaved from the resin with trifluoroacetic acid. Different peptide triazole conjugates with aryl, alkyl, and other functional groups were constructed (Table 2). The near quantitative conversion (>95% yield) of azide to 4-substituted triazoles was verified for all peptides by HPLC. The purified peptide triazoles were screened for gp120 binding with SPR.



Scheme 2. Construction of triazole and amide groups at the γ position of proline 6: a) CH_3CN/H_2O/DIEA/pyridine (4:4:2:1), R alkyne (5 equiv), and CuI (1 equiv), overnight; b) $P(CH_3)_3$, dioxane, H_2O, room temperature, 30 min; c) R^1 acid chloride, triethylamine in dichloromethane, 30 min. Fmoc = 9-fluorenylmethyloxycarbonyl, DIEA = *N*,*N*-diisopropylethylamine, SPPS = solid-phase peptide synthesis.

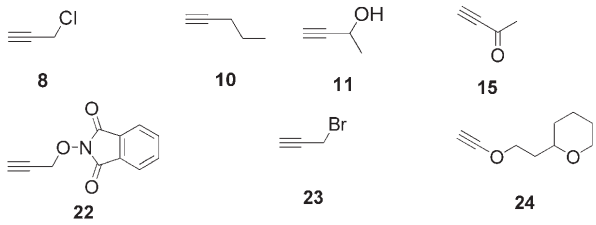
Table 2. Alkynes used in the [3+2] cycloaddition reaction and aryl groups used in amide coupling.[a]

Strong Binding R Groups (12–434 nM)



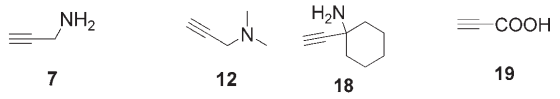
5 6 13 14

Weak Binding R Groups (3–100 μM)



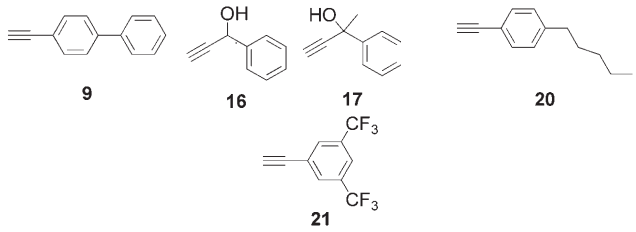
8 10 11 15 22 23 24

Nonbinding R Groups



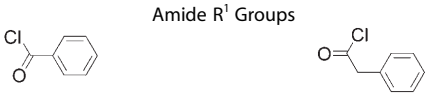
7 12 18 19

Nonspecific Binding R Groups



9 16 17 20 21

Amide R^1 Groups



25 26

[a] Compound numbers refer to the polypeptide conjugates produced with these alkyne or acid chloride precursors; see Scheme 2.

The peptide triazole conjugates that bind gp120 with the highest affinity have K_D values in the nanomolar range and are listed in Table 3. Peptides linked with other aromatic, aliphatic, or alternative functional groups bind gp120 with low affinity or nonspecifically. Peptide conjugates that bind nonspecifically or that have low solubility were not pursued for further characterization.

Among all peptide triazole conjugates shown in Table 2, the 4-phenyl-substituted triazole **5** binds to gp120 with high affinity ($K_D = 12.7$ nM). Among the four peptides with highest affinity, **5** had the highest on rate (k_a) and lowest off rate (k_d) (Table 3). Figure 1 shows the direct binding of peptide **5** to YU2 gp120. Solutions of increasing peptide concentration were

Table 3. Binding constants of triazole-modified peptides.[a]

Peptide	k_a [$M^{-1}s^{-1}$][b]	k_d [s^{-1}][c]	K_D [nM][d]	χ^2
5	3.62×10^5	4.59×10^{-3}	12.7	0.491
6	6.46×10^4	5.91×10^{-3}	91.8	1.6
13	2.09×10^4	9.07×10^{-3}	434	1.14
14	5.67×10^4	8.78×10^{-3}	155	0.173

[a] Determined by direct interaction with surface-immobilized YU2 gp120 in SPR. [b] Association rate constant. [c] Dissociation rate constant. [d] Equilibrium constant.

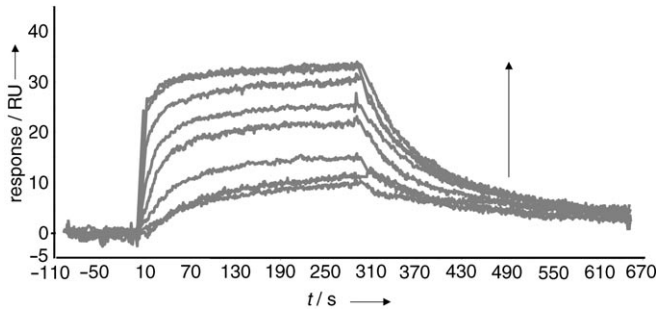


Figure 1. Direct binding of peptide **5** to immobilized YU2 gp120. The response sensorgrams were obtained with increasing concentrations of peptide **5** (10, 20, 50, 100, 250, 500 nM, and 1 μM as indicated by the arrow).

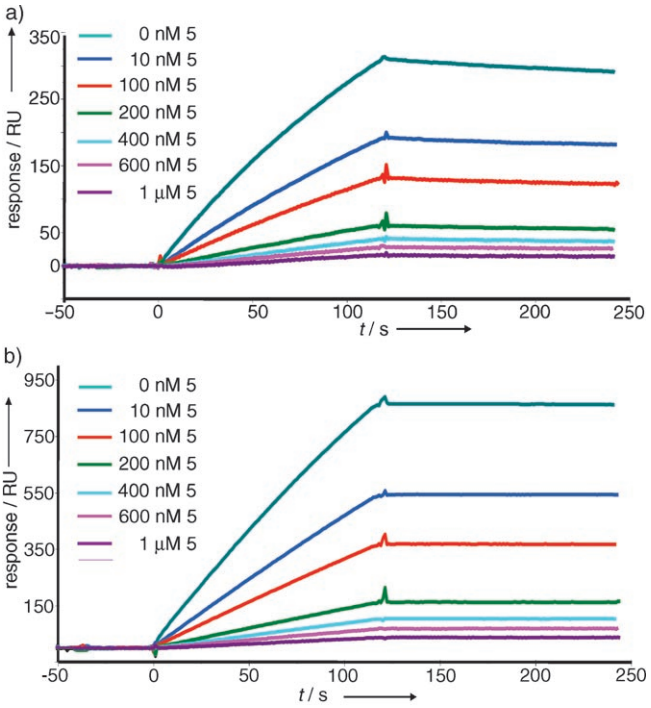


Figure 2. Inhibition of binding of YU2 gp120 to a) CD4 and b) 17b by peptide **5**. CD4 and 17b were immobilized on the biosensor chip surface, and YU2 gp120 (100 nM) was passed over the surfaces in solution with increasing concentrations of peptide **5** (from 10 nM to 1 μM, as indicated).

passed over a surface with the viral glycoprotein immobilized at high density (5000 RU). The equilibrium constant K_D was calculated from the global fit of direct binding curves by using the instrument software (Biaevaluation 3000). By using soluble CD4 (sCD4) as a standard, the R_{max} value of the peptide showed that the peptide binds to gp120 with a 1:1 stoichiometry.

To assess the inhibition of gp120 binding to sCD4 and 17b, YU2 gp120 (100 nM) as the analyte was passed over immobilized sCD4, 17b, and control 2B6R Fab in the absence or presence of peptide **5**. The peptide **5** exhibited no direct binding to sCD4, 17b, or control 2B6R. Figure 2 shows that increasing the concentration of peptide **5** from 10 nM to 1 μM leads to almost complete inhibition of gp120 binding to both sCD4- and 17b-coated surfaces. This effect was used to generate IC_{50} values, which were calculated by using the fraction of the initial rate (6–20 s) of YU2 gp120 binding in the presence over the absence of peptide **5** and plotting these against the log of peptide concentration.

In the survey of other derivatives with modifications at position 6 besides the high-efficacy derivatives in Table 3, we found that most were either relatively weak binders or bound nonspecifically. These results are summarized in Table 2. Peptides **9**, **16, 17**, **20**, and **21** bind to immobilized gp120 but in a manner independent of concentration. With **9** as an example, we found that this peptide fails to inhibit the interactions binding of gp120 to CD4 and to 17b even at concentrations up to 1 μM.

We carried out further experiments to evaluate the significance of triazole substitution at the γ position of proline by coupling the benzoyl group to γ-aminoproline (**25**). The purified peptide binds to immobilized YU2 gp120 with a K_D value in the same order of magnitude as that of the parent peptide **1**, even though the **25** contains an additional phenyl ring. Similar results were observed with peptide **26** (Table 2). These findings confirm that the presence of the phenyl ring in the 4-substituted triazole (peptide **5**) is critical in effecting high-affinity binding. We theorize that the triazole group formed with the configuration-constraining proline core may hold the phenyl ring in a more rigid orientation, which requires a smaller entropic penalty for gp120 binding than is the case with the amide phenyl rings in peptide **25** and **26**.

We also investigated the mode of by which the sCD4–gp120 interaction is blocked. The two most crucial residues on CD4 for gp120 binding are Phe 43 and Arg 59. The parent peptide **1** has been shown previously to block gp120 binding to CD4 and 17b through an allosteric noncompetitive mechanism.[9] Nonetheless, the presence of the phenyl ring on the triazole group in peptide **5** led us to consider the possibility that the conjugation confers **5** with the ability to bind competitively with CD4 in its binding pocket with Phe 43. To investigate this possibility, soluble CD4 at concentrations in the range of 0–2 μM was passed over a gp120-primed surface in the presence or absence of peptide **5** (1 μM). The binding capacity of sCD4 for gp120 was decreased over a wide range of sCD4 concentrations (data not shown), showing that the peptide binds to gp120 by a noncompetitive allosteric mode, similar to that observed with peptide **1**.[9]

Overall, peptide **5** strongly inhibits the binding of gp120 to both host cell receptors with potency close to the nanomolar

range. This observation evokes the enticing possibility of its use as a lead for the design of small-molecule antagonists of the HIV-1 fusion process. Because gp120 is relatively flexible in its unbound form,[11] it is likely that it is present in a range of conformations between its unbound and activated states. Peptide **5** could either stabilize a nonproductive intermediate conformation of gp120 or prevent its transition to an active conformation.

In summary, we have demonstrated that peptide **5**, with (2*S*,4*S*)-4-(4-phenyl-1*H*-1,2,3-triazol-1-yl)pyrrolidine-2-carboxylic acid at residue position 6, binds to gp120 with an affinity two orders of magnitude greater than that of the parent peptide **1** and strongly inhibits the interaction of gp120 with both CD4 and 17b. These results are encouraging for the potential use of peptide **5** as a lead tool in the drug-discovery process. Further mutations and truncations of peptide **5**, and different triazole conjugates of peptide **3** are currently being examined. This work establishes for the first time that azidoproline incorporated internally in a peptide sequence can be used effectively as a tool for side-chain conjugation through click chemistry, with consequent improvement in lead optimization.

Acknowledgments

This research was supported by a National Institute of Health (NIH) grant (PO1 GM 056550-08/C210JC). We thank Dr. Simon Cocklin, Dr. Tetsuya Ishino and Dr. Mauro Sergi for their technical advice.

Keywords: antiviral agents · click chemistry · HIV-1 · inhibitors · viruses

[1] a) V. V. Rostovtsev, L. G. Green, V. V. Fokin, K. B. Sharpless, *Angew. Chem.* **2002**, *114*, 2708 – 2711; *Angew. Chem. Int. Ed.* **2002**, *41*, 2596 – 2599; b) C. W. Tornoe, C. Christensen, M. Meldal, *J. Org. Chem.* **2002**, *67*, 3057 – 3064; c) H. C. Kolb, M. G. Finn, K. B. Sharpless, *Angew. Chem.* **2001**, *113*, 2056 – 2075; Angew . *Chem. Int. Ed. Engl.* **2001**, *40*, 2004 – 2021; d) H. C. Kolb, K. B. Sharpless, *Drug Discovery Today* **2003**, *8*, 1128.

[2] a) Q. Wang, T. R. Chan, R. Hilgraf, V. V. Fokin, K. B. Sharpless, M. G. Finn, *J. Am. Chem. Soc.* **2003**, *125*, 3192 – 3193; b) A. Deiters, T. A. Cropp, M. Mukherji, J. W. Chin, J. C. Anderson, P. G. Schultz, *J. Am. Chem. Soc.* **2003**, *125*, 11782 – 11783; c) A. J. Link, D. A. Tirrell, *J. Am. Chem. Soc.* **2003**, *125*, 11164 – 11165; d) A. E. Speers, B. F. Cravatt, *Chem. Biol.* **2004**, *11*, 535 – 546; e) F. Fazio, M. C. Bryan, O. Blixt, J. C. Paulson, C. H. Wong, *J. Am. Chem. Soc.* **2002**, *124*, 14397 – 14402; f) R. Manetsch, A. Krasinski, Z. Radic, J. Raushel, P. Taylor, K. B. Sharpless, H. C. Kolb, *J. Am. Chem. Soc.* **2004**, *126*, 12809; g) B. Helms, J. L. Mynar, C. J. Hawker, J. M. Frechet, *J. Am. Chem. Soc.* **2004**, *126*, 15020 – 15021.

[3] UNAIDS, World Health Organization **2003**.

[4] a) D. Klatzmann, E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J. C. Gluckman, L. Montagnier, *Nature* **1984**, *312*, 767 – 768; b) A. G. Dalgleish, P. C. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, R. A. Weiss, *Nature* **1984**, *312*, 763 – 767.

[5] a) D. C. Chan, D. Fass, J. M. Berger, P. S. Kim, *Cell* **1997**, *89*, 263 – 273; b) R. Wyatt, J. Sodroski, *Science* **1998**, *280*, 1884 – 1888; c) K. Tan, J.-h. Liu, J.-h. Wang, S. Shen, M. Lu, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 12303 – 12308.

[6] a) A. Trkola, T. Dragic, J. Arthos, J. M. Binley, W. C. Olson, G. P. Allaway, C. Cheng-Mayer, J. Robinson, P. J. Maddon, J. P. Moore, *Nature* **1996**, *384*, 184 – 187; b) Y. Feng, C. C. Broder, P. E. Kennedy, E. A. Berger, *Science* **1996**, *272*, 872 – 877; c) B. J. Doranz, J. Rucker, Y. Yi, R. J. Smyth, M. Samson, S. C. Peiper, M. Parmentier, R. G. Collman, R. W. Doms, *Cell* **1996**, *85*, 1149 – 1158; d) T. Dragic, V. Litwin, G. P. Allaway, S. R. Martin, Y. Huang, K. A. Nagashima, C. Cayanan, P. J. Maddon, R. A. Koup, J. P. Moore, W. A. Paxton, *Nature* **1996**, *381*, 667 – 673; e) L. Wu, N. P. Gerard, R. Wyatt, H. Choe, C. Parolin, N. Ruffing, A. Borsetti, A. A. Cardoso, E. Desjardin, W. Newman, C. Gerard, J. Sodroski, *Nature* **1996**, *384*, 179 – 183.

[7] a) C. E. Baldwin, R. W. Sanders, B. Berkhout, *Curr. Med. Chem.* **2003**, *10*, 1633 – 1642; b) P. F. Lin, W. Blair, T. Wang, T. Spicer, Q. Guo, N. Zhou, Y. F. Gong, H. G. Wang, R. Rose, G. Yamanaka, B. Robinson, C. B. Li, R. Fridell, C. Deminie, G. Demers, Z. Yang, L. Zadjura, N. Meanwell, R. Colonno, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 11013 – 11018.

[8] M. Ferrer, S. C. Harrison, *J. Virol.* **1999**, *73*, 5795 – 5802.

[9] A. C. Biorn, S. Cocklin, N. Madani, Z. Si, T. Ivanovic, J. Samanen, D. I. Van Ryk, R. Pantophlet, D. R. Burton, E. Freire, J. Sodroski, I. M. Chaiken, *Biochemistry* **2004**, *43*, 1928 – 1938.

[10] J. T. T. Lundquist, J. C. Pelletier, *Org. Lett.* **2002**, *4*, 3219 – 3221.

[11] S. A. Leavitt, A. Schön, J. C. Klein, U. Manjappara, I. M. Chaiken, E. Freire, *Curr. Protein Pept. Sci.* **2004**, *5*, 1 – 8.

Received: September 2, 2005
Published online on November 22, 2005

