Rapid and Quantitative Cyclization of Multiple Peptide Loops onto Synthetic Scaffolds for Structural Mimicry of Protein Surfaces

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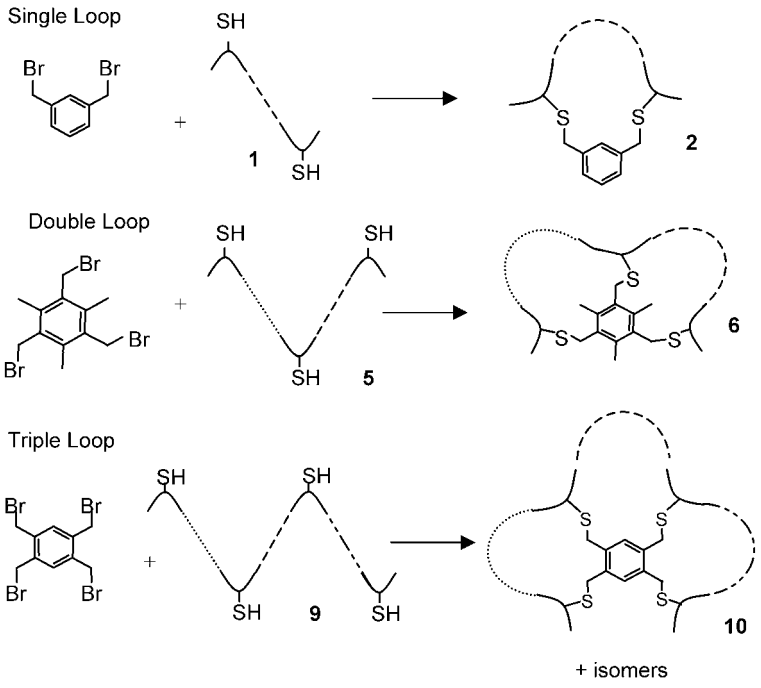
Structure-based design of synthetic peptide-based molecules that mimic the functional site of natural proteins, plays an important role in drug discovery nowadays.[1–5] Their application is widespread, ranging from synthetic antiviral,[3,6] antifertility,[1,2,7] or antitumor[2,7,8] agents to therapeutic agents that are able to mimic[9] or disrupt[4,10] protein–protein interactions. A variety of structural mimics exist for α-helices,[11,12] β-turns or hairpins,[11,13] and β-sheets.[11,14] However, more complex topologies, like four-helix bundles,[15] are often needed in order to mimic protein function adequately.[16] The total synthesis of such complex structures is generally demanding; this limits their application and emphasizes the need for high-efficiency synthetic strategies. In this communication, we describe a one-step procedure for the immobilization of (multiple) peptide loops onto a synthetic scaffold (Scheme 1) starting from a linear peptide. The reaction is extremely fast and clean and runs very well with linear peptides that are 2–30 amino acids long (> 30 not tested). It is compatible with all possible unprotected side-chain functionalities (except for free cysteine). It therefore avoids the need for complex synthetic strategies and this makes the reaction highly versatile with a very wide scope.

As part of our research program on the mapping and reconstruction of the discontinuous epitope of follicle-stimulating hormone (FSH),[17] which is a heterodimeric member of the cysteine-knot protein family,[18] we recently discovered the fast and quantitative cyclization of dicysteine-containing peptides upon their treatment with α,α′-dibromoxylenes (**T2**). In organic solvents such as ACN, the reaction is rather slow and unselective,[19] but it becomes unusually fast and entirely selective for cysteines when performed in aqueous solutions.[20] For example, treatment of a 0.5 mM solution of the peptide *CRVPGDAHHADSLC# (**1a**, where *=acetyl and #=amide) with 1.05 equiv of *m*-**T2** in a 1:7 mixture of ACN/NH_4HCO_3 (20 mM, pH 7.8) gives the corresponding monocyclic product **2a** with >80% yield in less than 15 min at RT (see Table 1). The corresponding intramolecular *SS*-dimer **3** is not formed (<5%) as oxidative cyclization is not competitive under these conditions. There is no doubt that the reaction takes place exclusively at the free sulfhydryl groups, since corresponding peptides without sulfhydryl groups[21] do not react at all with **T2** scaffolds in the solvent system used.



Scheme 1. *Schematic representation of the one-step synthesis of single-, double-, and triple-loop peptide constructs by treating di-, tri-, and tetracysteine containing peptides with bis-, tris-, and tetrakis(bromomethyl)-benzene derivatives.*

The difference in reactivity amongst various dicysteine-containing peptides that we have studied is negligible. The half-lives of peptides **1a–g** in the reaction with *m*-**T2** vary only slightly ($t_{1/2}$ = 1.4–3.0 min, see Table 1), despite the fact that their length (14–42) and the number of amino acids that separate the two cysteines (0–22) are very different. In sharp contrast to this, there is a large difference in reactivity amongst different scaffolds. *o*-**T2** (average $t_{1/2}$ = 1.4 min) is slightly more reactive than *m*-**T2** (average $t_{1/2}$ = 2.2 min), but both react much faster than *p*-**T2** (average $t_{1/2}$ = 8.6 min). Even with a fivefold excess of *m*-**T2** the reaction predominantly gives the monocyclic product **2**, whereas the 1:2 product **3** (Scheme 2) is formed with <10% yield.

Cyclization of dicysteine-containing peptides is a two-steps process: initial formation of linear intermediate **4** (Scheme 2) followed by intramolecular cyclization to give cyclic peptide **2**. All intermediates (*o*-**4**, *m*-**4**, and *p*-**4**) are highly reactive and cyclize rapidly at low concentration, but their reactivity does not follow that of the template itself. For example, intermediates *o*-**4** and *p*-**4** are the most reactive by far and can only be observed by HPLC for larger ring sizes (e.g., peptide **1f**), whereas *m*-**4** is significantly more stable and was observed in significant amounts (=15%) for most peptides (see Table 1). Most likely, intermediates *o*-**4** and *p*-**4** are activated by a stabilizing resonance-effect in which the sulfur of the first thioether bond activates the second bromomethyl group for nucleophilic attack.[22]

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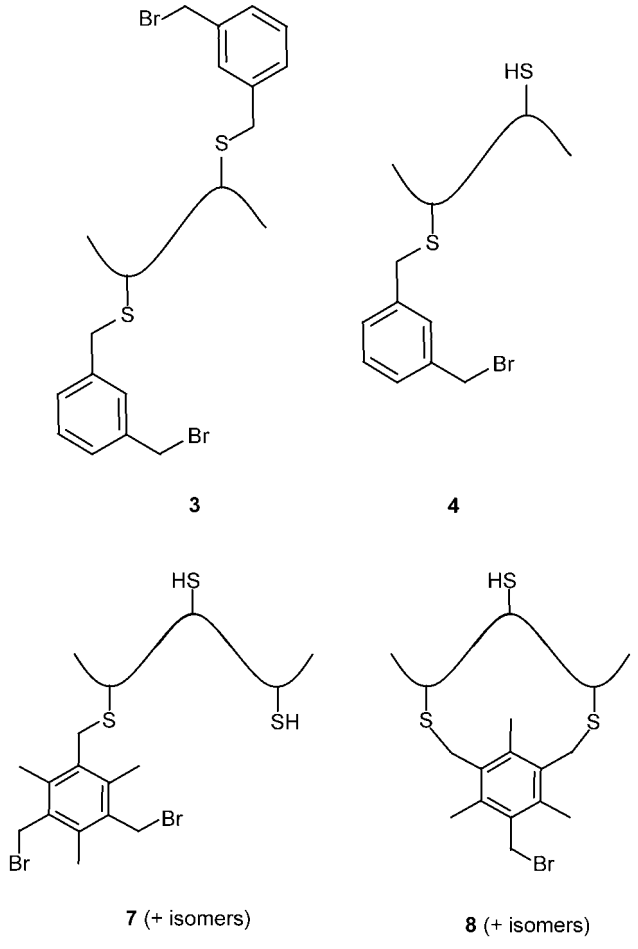
Table 1. *Product yields as a function of time in the treatment of peptides **1**, **5**, and **9** with 1.05 equiv of template.*

Peptide	Peptide Sequence[a]	template	% of products **2/6/10**[c] 1 min	5 min	15 min	45 min	$t_{1/2}$ [min]
1a	*CR_{62}VPGDAHHADSL_{73}C#	*o*-**T2**	33.9	71.0	84.8	86.2	1.7
		m-**T2**	9.3 (7.0)[b]	56.8	80.2	86.0	3.0
		p-**T2**	4.8	22.8	45.1	65.9	15.8
1b	*CR_{62}VPGAAHHADSL_{73}C#	*m*-**T2**	19.7 (10.3)[b]	71.2	94.2	97.6	2.5
1c	*V_{61}RCPGAAHHADSL_{73}C#	*m*-**T2**	23.0 (16.0)[b]	84.3 (1.5)[b]	94.6	95.4	1.5
1d	*V_{61}RVPGCAHCADSLY_{74}#	*m*-**T2**	38.4	84.5	93.8	93	1.6
1e	*V_{61}RVPGACCHADSLY_{74}#	*m*-**T2**	38.1 (1.0)[b]	83	94.8	99	1.4
1f	*CV_{57}YETVRVPGAAHHADSLYTYPV$_{78}$C#	*o*-**T2**	37.1 (5.8)[b]	82.3	95.6	93.9	1.0
		m-**T2**	16.1 (15.4)[b]	62.4 (4.3)[b]	91.5	93.2	1.8
		p-**T2**	31.3 (5.3)[b]	59.3	78.7	95.3	2.7
1g	*T_{46}KIQKTATFKELVYETCRVPGAAHHADSLCTYPVATQAHAGK$_{86}$#	*o*-**T2**	29.5	81.5	94.4	99.5	1.5
		m-**T2**	16.5 (5.9)[b]	63.0	84.5	87.8	1.8
		p-**T2**	13.3	34.7	59.2	90.1	7.4
5	*CA_{15}IEKEEARFAIS$_{21}$CT_{60}VRVPGAAHHADSLYT_{76}C#	**T3**	25.1 (11.2)[b]	86	91.2	91.2	1.2
9	*CA_{14}IEKEEARFAIS$_{21}$CGCT_{60}VRVPGAAHHADSLYT_{76}C#	**T4**	n.d.	n.d.	n.d.	~65	n.d.

[a] Numbers correspond to amino acid positions in the FSH β-chain; * = acetyl; # = amide. [b] Number in brackets is the amount (%) of intermediate **4** that was formed. [c] Yields were determined by HPLC-peak integration. [d] Small variations in yields are caused by the presence of impurities that do not react and therefore decrease the yield of final product. These have an R_f value similar to that of the linear peptide. n.d. = not determined.



Scheme 2. *Structure of the various intermediates that are formed in the peptide cyclization reactions.*

Treatment of linear peptides that contain three or four cysteines with either 2,4,6-tris(bromomethyl)mesitylene (**T3**) or 1,2,4,5-tetrabromodurene (**T4**) gives easy access to synthetic constructs that consist of two or three different peptide loops and are immobilized on the scaffold (Scheme 1). For example, treatment of a 0.5 mM solution of the tri-SH peptide **5** with 1.05 equiv of **T3** in 1:1 ACN/NH_4HCO_3 (40 mM, pH 7.8) gives the corresponding bicyclic product **6** with >90% yield. The reaction is extremely fast and runs to completion in less than 5 min to produce **6** as the sole product (regioisomers are not possible). The high reactivity of **T3**, and probably also that of the linear (**7**) and monocyclic (**8**) intermediates (Scheme 2), results from the electron-donating effect of the three additional methyl groups, since intermediates **7** and **8** are hardly detectable during cyclization. Similarly, treatment of a 0.5 mM solution of the tetra-SH peptide **9** with 1.05 equiv of **T4** in 1:1 ACN/NH_4HCO_3 (40 mM, pH 7.8) gives the corresponding tricyclic product **10**. The product is formed as a mixture of at least four different regioisomers in an overall yield of ~65%. Although the reaction with **T4** is somewhat slower than that with **T3**, it runs to completion within 30 min at RT.

In order to investigate to what extent cyclization of the peptides alters their binding properties, we synthesized an overlapping set of 12-mer FSH β-peptides, both linear and cyclized with *m*-**T2**, onto a solid support in microwell format (see Supporting Information). The overlapping peptides represent amino acids 1–12, 2–13, 3–14, etc., of the native protein chain (Pepscan method).[23] ELISA binding studies with a monoclonal antibody (mAb 2) specific for the FSH β-chain[17] showed highly selective binding at 10 μg mL^{-1} to the cyclic sequence *m*-**T2**[*CRVPGAAHHADSLC]-PS, which corresponds to the top of the β3-loop (Figure 1). No binding was observed for the corresponding linear peptide *RVPGAAHHADSL-PS (PS = polymeric support). Replacement analysis studies, in which all twelve amino acids in the loop were systematically substituted for the other 19 natural L-amino acids, clearly showed that the four amino acids R62, G65, D71, and L73 are essential for binding and do not resist substitution without a complete loss of the binding to mAb 2.[17] Binding studies in solution with Biacore

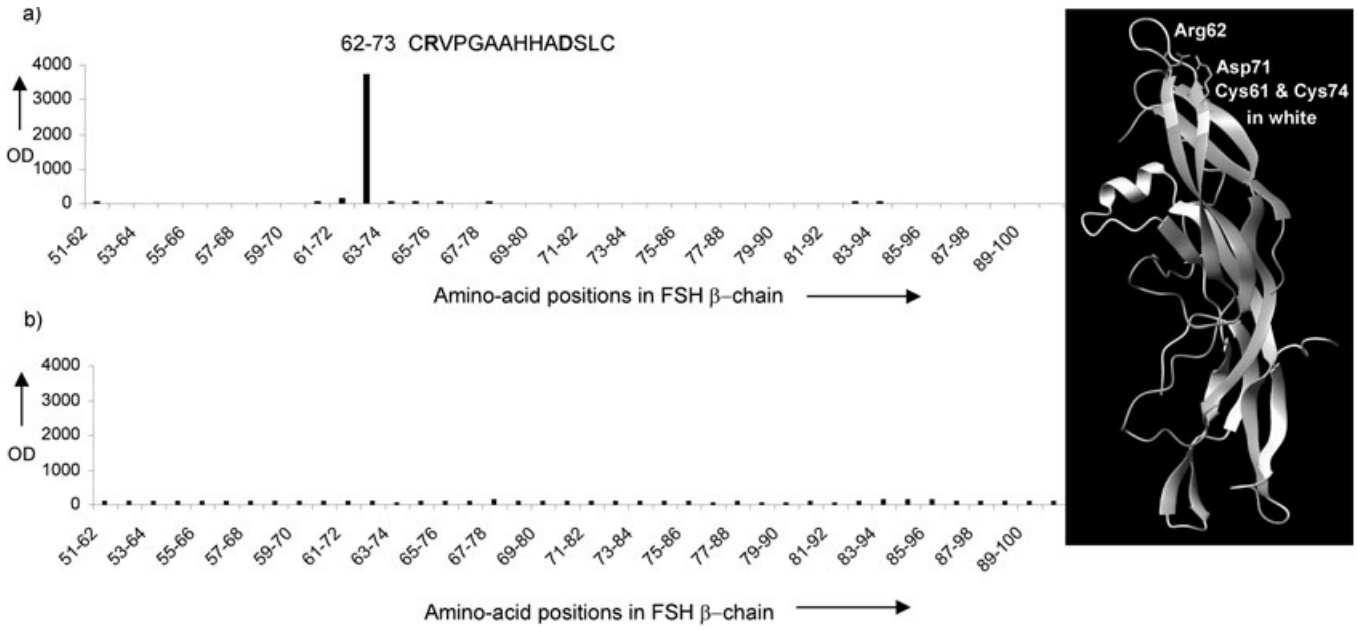


Figure 1. *ELISA binding data for a) 12-mer loopscan and b) the corresponding 12-mer linear Pepscan for the β-subunit of FSH (α-chain in white, β-chain in gray). The data show improved binding of peptide 62–73 (**C**RVPGAAHHADSL**C**) upon loop formation with the dibromoscaffold* m-***T2***. Amino acids Cys61 and Cys74 (side chains not shown) are indicated in white; Arg62 and Asp72 (side chains shown) are in dark gray. The figure was prepared with the program MOLMOL.*[24]

3000 have confirmed these results and showed a K_d of ~2–3 μM for the cyclic peptides *o*-**2b**, *m*-**2b**, and *p*-**2b**. No binding was observed for the linear peptide **1b** at 200 μM ($K_d \geq 1$ mM). Moreover, the cyclic SS-dimer of **1b** only showed a K_d of ~130 μM, which is two orders of magnitude lower than that of the corresponding cyclic peptide **2b**.

In conclusion we can state that the fast and quantitative cyclization of linear peptides with unprotected side-chain functionalities that have multiple free cysteines onto poly(bromomethyl)-functionalized synthetic scaffolds, provides easy access to an extremely diverse class of polycyclic peptide structures with interesting application in peptidomimetics.

Experimental Section

Characterization of cyclic peptide-constructs by ES-MS: *m*-**2a**: $[M]^{2+}$ = 813.1 (calcd 813.0), $[M]^{3+}$ = 542.4 (calcd 542.3); *m*-**4a**: $[M]^{2+}$ = 853.1 (calcd 853.4), $[M]^{3+}$ = 569.8 (calcd 569.2); *m*-**2b**: $[M]^{2+}$ = 790.5 (calcd 790.9), $[M]^{3+}$ = 527.4 (calcd 527.6); *m*-**4b**: $[M]^{2+}$ = 831.4 (calcd 831.4), $[M]^{3+}$ = 554.2 (calcd 554.2); *m*-**2c**: $[M]^{2+}$ = 790.5 (calcd 790.9); *m*-**4c**: $[M]^{2+}$ = 831.0 (calcd 831.4); *m*-**2d**: $[M]^{2+}$ = 818.0 (calcd 817.9); *m*-**2e**: $[M]^{2+}$ = 818.0 (calcd 817.9); *m*-**2f**: $[M]^{2+}$ = 1398.4 (calcd 1398.7), $[M]^{3+}$ = 932.5 (calcd 932.8); *m*-**4f**: $[M]^{2+}$ = 1438.4 (calcd 1439.1), $[M]^{3+}$ = 959.7 (calcd 959.7); *m*-**2g**: $[M]^{2+}$ = 1147.6 (calcd 1147.6), $[M]^{3+}$ = 918.2 (calcd 918.3), $[M]^{4+}$ = 765.5 (calcd 765.4); *m*-**4g**: $[M]^{2+}$ = 1167.8 (calcd 1168.0), $[M]^{3+}$ = 934.5 (calcd 934.4); **6**: $[M]^{3+}$ = 1183.3 (calcd 1183.4), $[M]^{4+}$ = 888.6 (calcd 887.8); **10**: $[M]^{3+}$ = 1226.6 (calcd 1226.8), $[M]^{4+}$ = 920.0 (calcd 920.3).

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Keywords: cyclization · epitope mapping · peptides · peptidomimetics · protein models

[1] a) R. J. Aitken, *Expert Opin. Biol. Ther.* **2003**, *3*, 829–841; b) R. J. Aitken, *J. Reprod. Immunol.* **2002**, *57*, 273–287; c) K. Viswanath, P. Kirbat, *Econ. Polit. Wkly.* **2000**, *35*, 718–725.
[2] G. P. Talwar, *Immunol. Cell Biol.* **1997**, *75*, 184–189.
[3] R. H. Meloen, K. Dalsgaard, J. I. Casal, J. P. M. Langeveld, *Vaccine* **1995**, *13*, 885–886.
[4] H.-T. Zhang, Q. Wang, M. I. Greene, R. Murali, *Drug News Perspect.* **2000**, *13*, 325–329.
[5] For reviews on this topic, see: a) R. Jain, J. T. Ernst, O. Kutzki, H. S. Park, A. D. Hamilton, *Mol. Divers.* **2004**, *8*, 89–100; b) V. J. Hruby, *Nat. Rev. Drug Discovery* **2002**, *1*, 847–858; c) A. G. Cochran, *Curr. Opin. Chem. Biol.* **2001**, *5*, 654–659; d) L. L. Kiesling, J. E. Gestwicki, L. E. Strong, *Curr. Opin. Chem. Biol.* **2000**, *4*, 696–703; e) M. W. Peczuh, A. D. Hamilton, *Chem. Rev.* **2000**, *100*, 2479–2494.
[6] a) R. Sundaram, M. P. Lynch, S. V. Rawale, Y. Sun, M. Kazanji, P. T. Kaumaya, *J. Biol. Chem.* **2004**, *279*, 24141–24151; b) D. Ziegler, P. Fournier, G. A. H. Berbers, H. Steuer, K.-H. Wiesmueller, B. Fleckenstein, F. Schneider, G. Jung, C. C. King, C. P. Muller, *J. Gen. Virol.* **1996**, *77*, 2479–2489; c) J. P. M. Langeveld, J. I. Casal, A. D. M. E. Osterhaus, E. Cortés, R. de Swart, C. Vela, K. Dalsgaard, W. C. Puijk, W. M. M. Schaaper, R. H. Meloen, *J. Virol.* **1994**, *68*, 4506–4513; d) J. P. M. Langeveld, J. I. Casal, E. Cortés, G. van de Wetering, R. S. Boshuizen, K. Dalsgaard, W. M. M. Schaaper, R. H. Meloen, *Vaccine* **1994**, *12*, 1473–1480.
[7] R. H. Meloen, J. A. Turkstra, H. Lankhof, W. C. Puijk, W. M. M. Schaaper, G. Dijkstra, C. J. G. Wensing, H. B. Oonk, *Vaccine* **1994**, *12*, 741–746.
[8] a) S. A. Butler, E. M. Staite, R. K. Iles, *Oncol. Res.* **2003**, *14*, 93–100; b) P. L. Iversen, D. V. Mourich, H. M. Moulton, *Curr. Opin. Mol. Ther.* **2003**, *5*, 156–160; c) H. M. Moulton, P. H. Yoshihara, D. H. Mason, *Clin. Cancer Res.* **2002**, *8*, 2044–2051.
[9] a) L. Martin, F. Stricher, D. Misse, F. Sironi, M. Pugniere, P. Barthe, R. Prado-Gotor, I. Freulon, X. Magne, C. Roumestand, A. Menez, P. Lusso, F. Veas, C. Vita, *Nat. Biotechnol.* **2003**, *21*, 71–76; b) J. T. Ernst, O. Kutzki, A. K. Debnath, S. Jiang, H. Lu, A. D. Hamilton, *Angew. Chem.* **2002**, *114*, 288–291; *Angew. Chem. Int. Ed.* **2002**, *41*, 278–281; c) N. C. Wrighton, F. X. Farrell, R. Chang, A. K. Kashyap, F. P. Barbone, L. S. Mulcahy, D. L. Johnson, R. W. Barrett, L. K. Jolliffe, W. J. Dower, *Science* **1996**, *273*, 458–463; d) M. D. Struthers, R. P. Cheng, B. Imperiali, *Science* **1996**, *271*, 342–

345; e) B. Li, J. Y. K. Tom, D. Oare, R. Yen, W. J. Fairbrother, J. A. Wells, B. C. Cunningham, *Science* **1995**, *270*, 1657 – 1660.
[10] a) B.-W. Park, H.-T. Zhang, C. Wu, A. Berezov, X. Zhang, R. Dua, Q. Wang, G. Kao, D. M. O'Rourke, M. I. Greene, R. Murali, *Nat. Biotechnol.* **2000**, *18*, 194 – 198; b) M. A. Blaskovich, Q. Lin, F. L. Delarue, J. Sun, H. Soon Park, D. Coppola, A. D. Hamilton, S. M. Sebti, *Nat. Biotechnol.* **2000**, *18*, 1065 – 1070; c) W. Takasaki, Y. Kajino, R. Murali, M. I. Greene, *Nat. Biotechnol.* **1997**, *15*, 1266 – 1270.
[11] J. Venkatraman, S. C. Shankaramma, P. Balaram, *Chem. Rev.* **2001**, *101*, 3131 – 3152.
[12] I. Karl, *Acc. Chem. Res.* **1999**, *32*, 693 – 701.
[13] a) A. G. Cochran, N. J. Skelton, M. A. Starovasnik, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 5578 – 5583; b) K. Burgess, *Acc. Chem. Res.* **2001**, *34*, 826 – 835; c) M. Favre, K. Moehle, L. Jiang, B. Pfeiffer, J. A. Robinson, *J. Am. Chem. Soc.* **1999**, *121*, 2679 – 2685.
[14] J. S. Nowick, *Acc. Chem. Res.* **1999**, *32*, 287 – 296.
[15] a) R. Blake Hill, D. P. Raleigh, A. Lombardi, W. F. DeGrado, *Acc. Chem. Res.* **2000**, *33*, 745 – 754; b) M. Mutter, G. G. Tuchscherer, C. Miller, K. H. Altmann, R. I. Carey, D. F. Wyss, A. M. Labhardt, J. E. Rivier, *J. Am. Chem. Soc.* **1992**, *114*, 1463 – 1470.
[16] U. Reineke, R. Sabat, R. Misselwitz, H. Welfle, H. D. Volk, J. Schneider-Mergener, *Nat. Biotechnol.* **1999**, *17*, 271 – 275.
[17] P. Timmerman, E. van Dijk, W. Puijk, W. Schaaper, J. Slootstra, S. J. Carlisle, J. Coley, S. Eida, M. Gani, T. Hunt, P. Perry, G. Piron, R. H. Meloen, *Mol. Divers.* **2004**, *8*, 61 – 77.
[18] a) K. M. Fox, J. A. Dias, P. van Roey, *Mol. Endocrinol.* **2001**, *15*, 378 – 389; b) M. P. Rose, R. E. Gaines Das, A. H. Balen, *Endocr. Rev.* **2000**, *21*, 5 – 22.
[19] a) J. M. Benito, M. Meldal, *QSAR Comb. Sci.* **2004**, *23*, 117 – 129; b) M. A. Walker, T. Johnson, *Tetrahedron Lett.* **2001**, *42*, 5801 – 5804; c) Z. Szewczuk, K. L. Rebholz, D. H. Rich, *Int. J. Pept. Protein Res.* **1992**, *40*, 233 – 242; d) D. S. Kemp, P. McNamara, *Tetrahedron Lett.* **1981**, *22*, 4571 – 4574.
[20] P. Timmerman, J. Beld, W. C. Puijk, J. W. Slootstra, R. H. Meloen, EP 03075597.9/P62686EP00.
[21] The peptide *TFKELVYETARVPGAAHHADSLYTYPVATQ#, containing the amino acids D, E, H, K, Q, R, S, T, and Y, which have more or less nucleophilic characteristics, did not react with *m*-**T2** after 45 min under the reaction conditions used.
[22] G. Hennrich, V. M. Lynch, E. V. Anslyn, *Chem. Commun.* **2001**, 2436 – 2437.
[23] H. M. Geysen, R. H. Meloen, S. J. Barteling, *Proc. Natl. Acad. Sci. USA* **1984**, *81*, 3998 – 4002.
[24] R. Koradi, M. Billeter, K. Wüthrich, *J. Mol. Graphics* **1996**, *14*, 51 – 55.

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