Rolling Loop Scan: An Approach Featuring Ring-Closing Metathesis for Generating Libraries of Peptides with Molecular Shapes Mimicking Bioactive Conformations or Local Folding of Peptides and Proteins**

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Nature has found many intriguing and elegant ways to reduce the flexibility of peptides and proteins in order to increase both affinity and selectivity with an interacting partner molecule. Apart from the size of a peptide or protein, reduction of the flexibility by disulfide bridges is probably the most well known way to control shape.[1] Even more sophisticated modes for reducing the flexibility include sulfide bridges, zinc fingers, and multiple side chain knotting.[2–4] Often the affinity and/or biological activity is lost or at least significantly reduced upon breakage or absence of these conformational constraints. The principle of reducing flexibility—thereby increasing the affinity for a receptor by limiting the unfavorable entropy loss upon binding—is one of the most widely applied principles in drug development.

It is quite a challenge to reduce flexibility or to mimic local folding of peptides or (part of) proteins. Often the biologically active conformation of a peptide is not known or no longer present if only a small part of the protein—that is, a peptide—is studied. Clearly, ways for "probing" the biologically active conformation, approaches for creating alternative bioactive conformations, and possibilities for mimicking the spatial structure of parts of proteins could well lead to new insights into structure–biological activity relationships of peptides and proteins. Furthermore, this could also lead to new biologically interesting peptidomimetics to be used, for example, for interference with undesired disease-causing peptide–protein or protein–protein interactions.

We are therefore interested in developing general approaches for the introduction of conformational constraints into peptides without compromising the side chains of the amino acid residues, since these are often important for interaction with, for example, a receptor. In addition, we want to be able to introduce a conformational constraint, in principle, at any desired position of a peptide and of any desired size. The resulting procedure is therefore amenable to combinatorial approaches.

The approach is schematically depicted in Figure 1. The important characteristics are immediately apparent: 1) The loops connect two peptide–amide moieties, and thus do not compromise the α-carbon side chains; 2) loops can be formed by connecting any two desired peptide–amide bonds, thereby forming loops of any desired size; and 3) the loops "roll" over a peptide sequence, thereby "scanning" local conformations. We have therefore coined this approach the "rolling loop scan".

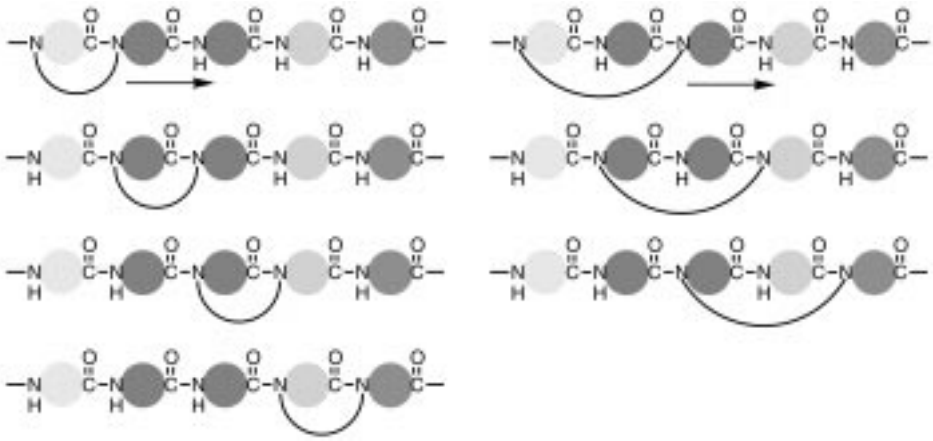
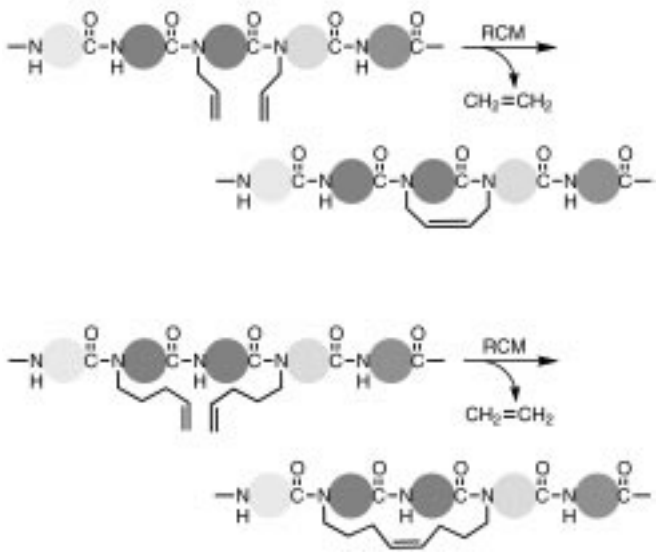


Figure 1. Schematic representation of the "rolling loop scan".

In this approach the loops were prepared by ring-closing metathesis (RCM, Scheme 1).[5] This is a particularly attrac-



Scheme 1. Connecting the side chains of N-alkylated peptides by ring-closing metathesis (RCM).

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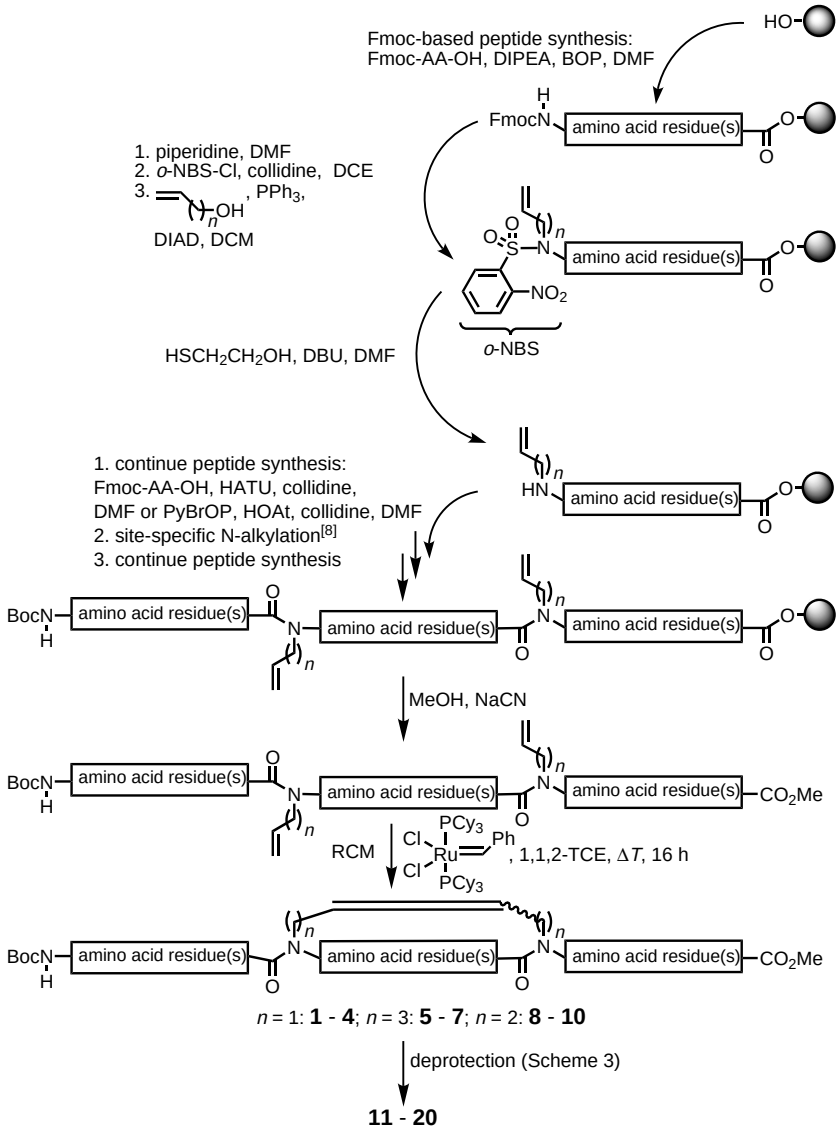
tive method, since the yields of RCM are usually high and no protection of the connecting side chains is required.[6, 7]

For the preparation of the bis-N-alkylated peptides—containing alkene side chains on the N-atoms of peptide–amide bonds—required for RCM a versatile solid-phase procedure was developed based on our site-specific N-alkylation of peptides, which enabled introduction of a side chain at any desired peptide–amide bond (Scheme 2).[8] First, a peptide was synthesized by the Fmoc strategy until the position was reached where the loop had to start. Then, the Fmoc group was removed and substituted by an *o*-nitrobenzenesulfonyl (*o*-NBS) group, which increases the acidity of the NH proton so that the amide became susceptible for a Mitsunobu reaction with an appropriate unsaturated alcohol. After removal of the *o*-NBS group, peptide synthesis was continued until the other end of the loop was reached. The second alkene side chain was introduced as described, and peptide synthesis was continued until the end of the peptide was reached. Ring-closing metathesis was carried out after cleavage from the resin, leading to the cyclized peptide.[9]

To demonstrate the principle of the rolling loop scan Leu-enkephalin was chosen. However, before applying this procedure one major hurdle had to be overcome. It was found that, depending on the number of amide bonds involved in the preparation of the loop, certain rules concerning the length of the alkene substituent had to be obeyed for RCM to take place:[10] 1) If two amide bonds are involved in the loop *N*-allylamides can be used in the starting bis-alkylated peptide ($n = 1$); 2) if three amides are involved in the loop *N*-pentenylamides have to be used ($n = 3$); 3) if four or more amide bonds are involved in the loop, *N*-homoallylamides will work ($n = 2$).

Obeying these rules a rolling loop scan of Leu-enkephalin was now carried out. All possible loops of Leu-enkephalin were prepared by RCM of the appropriate bis-*N*-alkylated amides according to the general procedure depicted in Scheme 2. The thus synthesized loop-containing Leu-enkephalin derivatives **1**–**10** were deprotected to give **11**–**20**, which were purified by preparative HPLC (Scheme 3). From these structures the "rolling" of the loops is evident (for example going from structures **11** to **14** and from structures **15**



Scheme 2. General solid-phase procedure for preparation of the cyclization precursors for the loop synthesis by RCM. AA = amino acid, Boc = *tert*-butoxycarbonyl, BOP = benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate, DCE = 1,2-dichloroethane, DCM = dichloromethane, DIAD = diisopropyl azodicarboxylate, DIPEA = ethyldiisopropylamine, DMF = dimethylformamide, Fmoc = 9-fluorenylmethoxycarbonyl, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOAt = 1-hydroxy-7-azabenzotriazole, *o*-NBS = *o*-nitrobenzenesulfonyl, PyBrOP = bromotripyrrolidinophosphonium hexafluorophosphate, 1,1,2-TCE = 1,1,2-trichloroethane.

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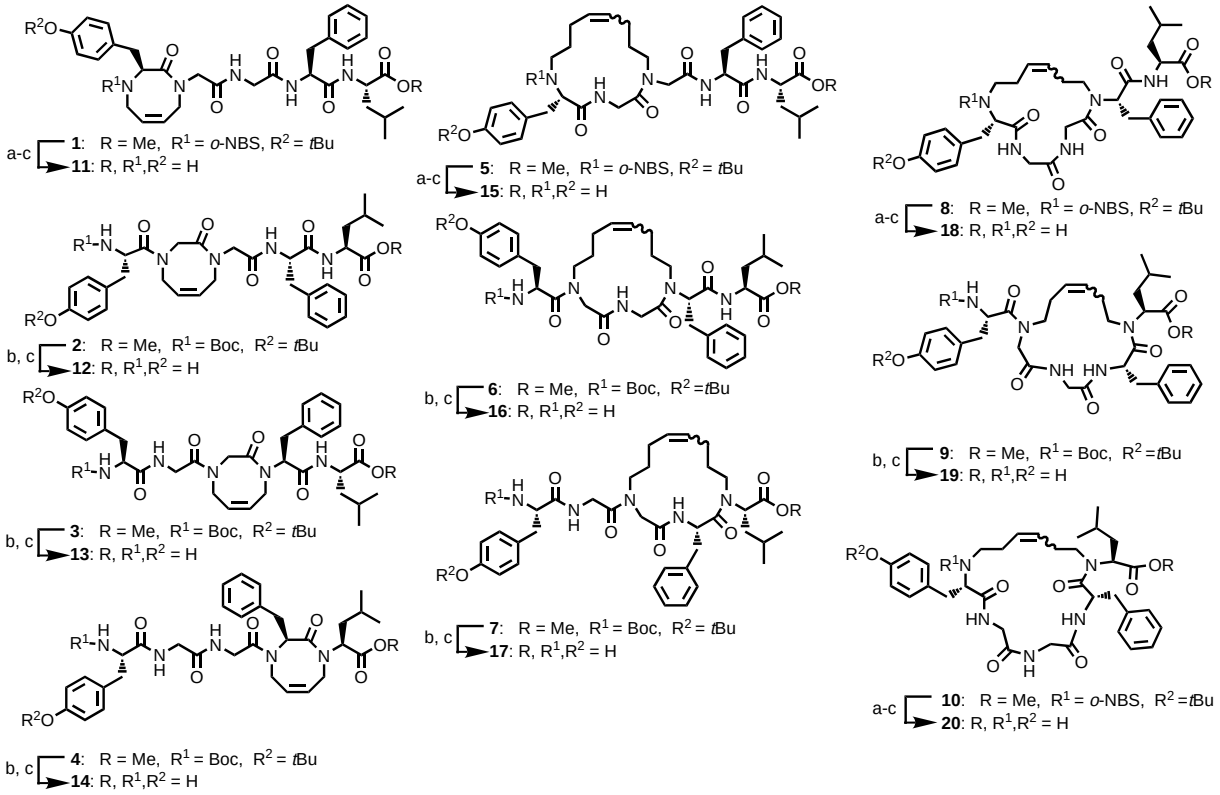

Scheme 3. All possible loop-containing Leu-enkephalin derivatives obtained by RCM. a) PhSH, K_2CO_3, MeCN; b) Tesser's base;[11] c) trifluoroacetic acid.

to **17**). The solid-phase synthesis was extremely efficient as is apparent from the overall yields and the calculated yield per step (in most cases >90%, Table 1). The yields of the RCM were more variable, but were quite satisfactory (≈50%) to good (>70%) and in some cases very good (>80%). The final deprotection was somewhat disappointing, but still the yields were quite satisfactory after preparative HPLC (Table 1).[11, 12]

An important aspect is the characterization and structural studies of these compounds. The use of mass spectrometry was quite essential. With ESI-MS the expected molecular ions were observed in all cases. The use of FAB-MS-MS was especially fruitful. It provided additional proof for the presence as well as the position of the loop (Figure 2). It was observed that the loop-containing enkephalin derivatives gave rise to ions which resulted in most cases from successive loss of C-terminal residues. However, this fragmentation occurred until the loop was reached, providing unequivocal proof for the position of the loop in the peptide. In general both ^{1}H (300 and 600 MHz) and ^{13}C NMR spectra of the loop-containing Leu-enkephalin derivatives were very complicated due to the presence of several conformers. In addition to peptide–amide rotamers other conformers were present which—probably because of the presence of the loop—were sufficiently stable at room temperature to be observed on the NMR time scale.

In conclusion, we have developed a general and versatile procedure for the synthesis of bis-N-alkylated peptides, from which loops can be formed by ring-closing metathesis. We think that this procedure is generally applicable to any

Table 1. Yields of the Leu-enkephalin derivatives **1**–**20** as well as exact masses of Leu-enkephalin derivatives **11**–**20**.

	Yield [%]				Yield [%]		Exact mass	
	overall[a]	per step[b]	RCM[c]		overall[d]	per step[b]	found	calcd
1	56	96	49	**11**	5.1	86	608.3069	608.3084
2	40	94	54	**12**	2.4	82	608.3058	608.3084
3	68	97	98	**13**	2.6	82	608.3094	608.3084
4	25	91	48	**14**	2.8	83	608.3064	608.3084
5	54	96	72	**15**	6.5	87	664.3723	664.3710
6	43	95	87	**16**	2.6	83	664.3718	664.3710
7	43	94	77	**17**	3.6	84	664.3733	664.3710
8	28	92	46	**18**	2.3	82	636.3403	636.3397
9	32	93	91	**19**	3.0	83	636.3416	636.3397
10	16	89	41	**20**	1.1	79	636.3422	636.3397

[a] The overall yield of the solid-phase synthesis. These yields may appear low, but are in fact quite high in view of the number of reactions (15) in this multistep synthesis. [b] Average yield per step. [c] Yield of the ring-closing metathesis. [d] Determined after purification by preparative HPLC.

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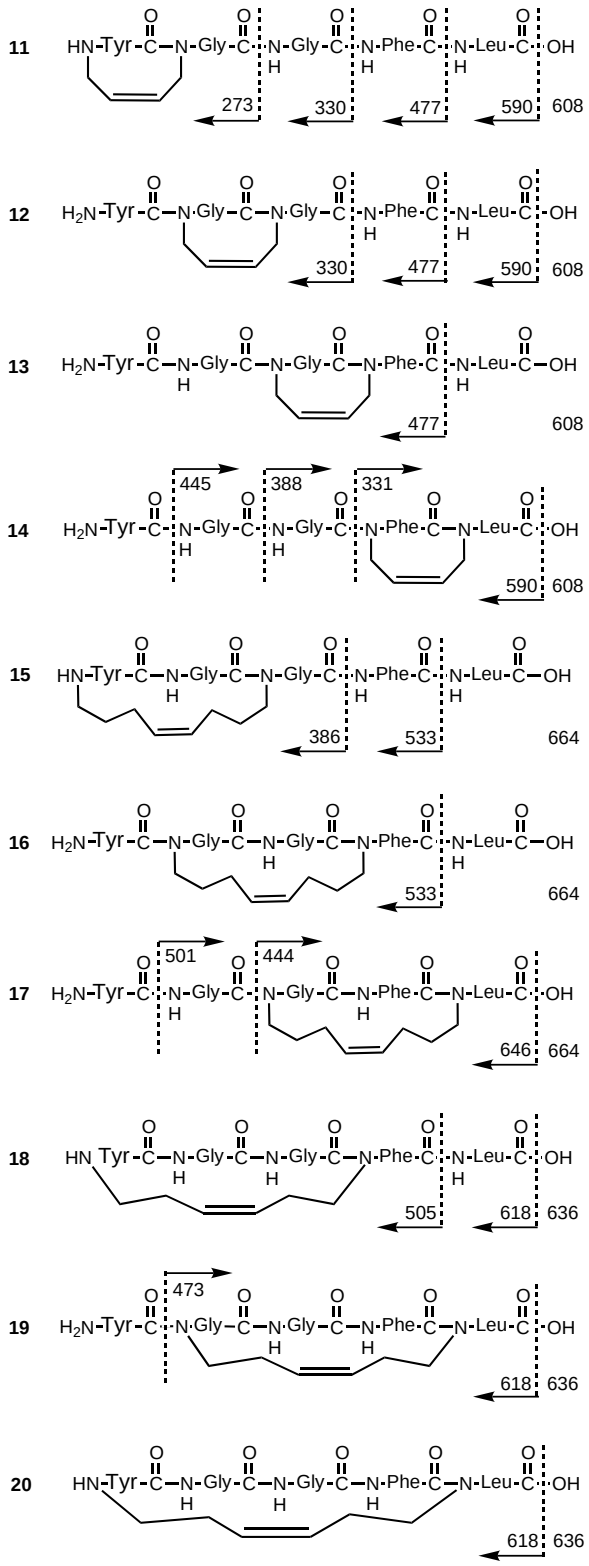

Figure 2. FAB-MS-MS fragments of Leu-enkephalin derivatives **11** – **20**.

peptide. Since all possible loops of a particular peptide can be formed, this rolling loop scan can be considered as a combinatorial approach towards generating libraries of (bioactive) conformers.

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[1] The presence of a number of disulfide bridges may give rise to formation of "cystine knots", providing absolute control over the three-dimensional structure. For a cystine knot in nerve growth factor, see N. Q. McDonald, R. Lapatto, J. Murray-Rust, J. Gunning, A. Wlodawer, T. L. Blundell, *Nature* **1991**, *354*, 411 – 414.

[2] Sulfide bridges are present in lantibiotics; see, for example, D. Kaiser, R. W. Jack, G. Jung, *Pure Appl. Chem.* **1998**, *70*, 97 – 104.

[3] For zinc fingers, see, for example, R. Kaptein, *Curr. Opin Struct. Biol.* **1991**, *1*, 63 – 70; R. Kaptein, *Curr. Opin Struct. Biol.* **1992**, *2*, 109 – 115.

[4] A preeminent example of multiple side chain knotting is found in the vancomycin antibiotics; recent review: D. H. Williams, B. Bardsley, *Angew. Chem.* **1999**, *111*, 1264 – 1286; *Angew. Chem. Int. Ed.* **1999**, *38*, 1172 – 1193.

[5] Recent reviews: R. H. Grubbs, S. Chang, *Tetrahedron* **1998**, *54*, 4413 – 4450; S. K. Armstrong, *J. Chem. Soc. Perkin Trans. 1* **1998**, 371 – 388.

[6] For RCM involving side chains of amino acids, see a) S. J. Miller, R. H. Grubbs, *J. Am. Chem. Soc.* **1995**, *117*, 5855 – 5856; b) S. J. Miller, H. E. Blackwell, R. H Grubbs, *J. Am. Chem. Soc.* **1996**, *118*, 9606 – 9614; c) R. H. Grubbs, H. E. Blackwell, *Angew. Chem.* **1998**, *110*, 3469 – 3472; *Angew. Chem. Int. Ed.* **1998**, *37*, 3281 – 3284; d) A. S. Ripka, R. S. Bohacek, D. H. Rich, *Bioorg. Med. Chem. Lett.* **1998**, *8*, 357 – 360; e) J. Pernerstorfer, M. Schuster, S. Blechert, *Chem. Commun.* **1997**, 1949 – 1950; f) B. E. Fink, P. R. Kym, J. A. Katzenellenbogen, *J. Am. Chem. Soc.* **1998**, *120*, 4334 – 4344; g) T. D. Clark, M. R. Ghadiri, *J. Am. Chem. Soc.* **1995**, *117*, 12364 – 12365.

[7] As far as we know there is only one example in the literature of cyclization of *N*-alkene substituents.[6a,b]

[8] J. F. Reichwein, R. M. J. Liskamp, *Tetrahedron Lett.* **1998**, *39*, 1243 – 1246.

[9] Although RCM can be carried out on the solid phase, so far higher yields were obtained if RCM was carried out in solution.

[10] The studies which led to these rules will be published elsewhere. Not all sizes of loops are allowed, since in addition to steric factors imposed by the side chains, a peptide – amide bond usually assumes a *trans*-like conformation: J. F. Reichwein, C. Versluis, R. M. J. Liskamp, unpublished results.

[11] Saponification of the methyl ester using Tesser's base (G. I. Tesser, I. C. Balvert-Geers, *Int. J. Pept. Protein Res.* **1975**, *7*, 295 – 305) is probably too harsh. Better results were obtained using the milder conditions of Corey et al. (E. J. Corey, I. Szekely, C. S. Shiner, *Tetrahedron Lett.* **1977**, 3529 – 3532).

[12] Compounds **5** – **10** were obtained as *cis*/*trans* mixtures, which so far could not easily be separated by HPLC.

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