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Reversible Backbone Protection Enables Combinatorial Solid-Phase Ring-Closing Metathesis Reaction (RCM) in Peptides

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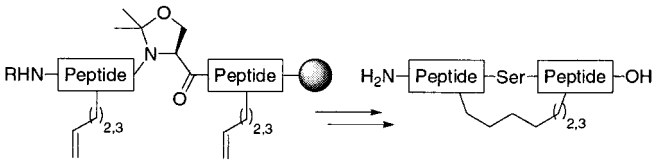
ABSTRACT



Attempts were made to apply the ring-closing metathesis reaction (RCM) to resin-bound peptides with olefinic side chains of different lengths. In a protein-derived homodetic 10mer peptide epitope the RCM reaction did not take place at all. Only by the introduction of the secondary structure disrupting reversible backbone protection Ser($\Psi^{Me,Me}$pro) and subsequent optimized reduction and purification protocols were we able to generate a full set of RCM cyclized peptides.

Protein−protein interactions are interesting targets for the development of novel therapeutic agents. The goal is to find a rigidified binding epitope that allows structure elucidation of the bioactive conformation. Since this molecule may then be used as a lead structure for further reductions in size, the efficient synthesis of such ligands is a crucial step in the rational development of nonpeptidic inhibitors for protein−protein interactions. One of the most promising strategy is the stepwise reduction of length of the epitope and subsequent attempts to rigidify the biological active conformation by cyclization.[1] The solid-phase ring-closing metathesis reaction (RCM) applying the Grubbs catalyst **1**[2] has been used for the tethering of preformed secondary structures in peptides such as β-turns[3] and α-helices.[4] In our attempts to further shorten and rigidify a recently identified 10-mer epitope-peptide,[5] serving as a receptor binding domain mimic derived from the serine protease urokinase-type Plasminogen Activator (uPA),[6] we applied the RCM side-chain cyclization concept to solid-phase bound peptides.[7] Similar to the solid-phase synthesis of β-turns by RCM, as previously described by Grubbs et al., the formation of the model peptide **3** (Figure 1) proceeded with good conversion rates,[8] whereas other cyclization positions in **3** and the *N*- and/or *C*-terminal elongated solid-phase bound peptides resulted in <5% RCM reaction products after 12 h.

(1) Kessler, H. *Angew. Chem., Int. Ed. Engl.* **1982**, *21*, 512−523.
(2) Schwab, P.; France, M. B.; Ziller, J. W.; Grubbs, R. H. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 2039−2041.
(3) (a) Miller, S. J.; Blackwell, H. E.; Grubbs, R. H. *J. Am. Chem. Soc.* **1996**, *118*, 9606−9614. (b) Jarvo, E. R.; Copeland, G. T.; Papaioannou, N.; Bonitatebus, J. P. J., Jr.; Miller, S. J. *J. Am. Chem. Soc.* **1999**, *121*, 11638−11643.
(4) Schafmeister, C. E.; Po, J.; Verdine, G. L. *J. Am. Chem. Soc.* **2000**, *122*, 5891−5892.
(5) Sequence: H-Ser[1]-Asn-Lys-Tyr-Phe-Ser-Asn-Ile-His-Trp[10]-OH (corresponding to amino acids uPA_{21-30}).
(6) Schmiedeberg, N.; Bürgle, M.; Wilhelm, O. G.; Lottspeich, F.; Graeff, H.; Schmitt, M.; Magdolen, V.; Kessler, H. *Peptides for the New Millenium*, Proceedings of the 16th American Peptide Symposium, Minneapolis, 1999; Fields, G. B., Tam, J. P., Barany, G., Eds.; Kluwer: Dordrecht, 2000; pp 543−545.
(7) Automated peptide synthesis was performed via Fmoc/*t*Bu strategy on tritylpolystyrene resin as described in Magdolen, V.; Bürgle, M.; Arroyo de Prada, N.; Schmiedeberg, N.; Riemer, C.; Schroeck, F.; Kellermann, J.; Degitz, K.; Wilhelm, O. G.; Schmitt, M.; Kessler, H. *Biol. Chem.* **2001**, *382*, 1197−1205.
(8) In a typical RCM reaction, about 130 mg of peptidyl resin (0.17 mmol/g) was suspended in 4 mL of dry DCM under an atmosphere of argon, 30 mg of **1** (0.04 mmol) was added in one portion, and the mixture was shaken at room temperature or refluxed for 12 h. Afterwards the resin was filtered and washed twice with DCM and methanol.

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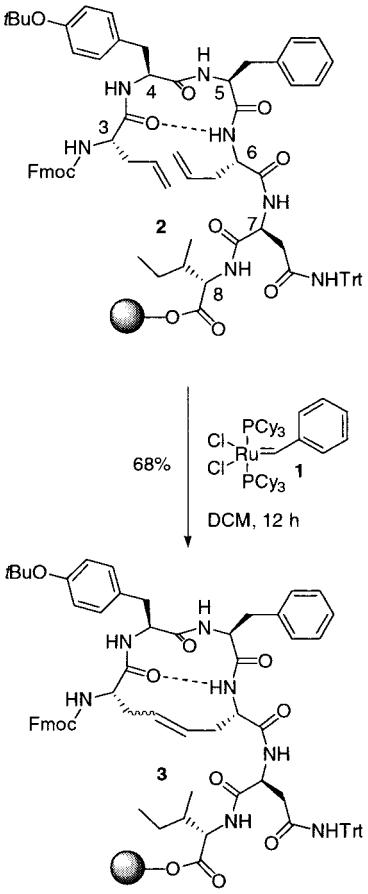

Figure 1. Solid-phase RCM reaction of peptidyl resin **2**.

The finding that the poor reactivity of these model peptides is not affected by the ring size, experimental conditions and stereochemical orientations, or positions or the number of carbon atoms of the olefinic side chains indicated that the formation of inappropriate secondary structural elements is preventing the peptides from the cyclization reactions.[9]

Subsequent examination of the few reports about solid-phase RCM in peptides with more than five amino acids[10] seemed to corroborate this suspicion, since all published peptide cycles contain proline or *N*-alkyl residues within their sequence. Since these modifications are assumed not to be tolerated in our target molecule, we considered the strategy of reversible backbone protection[11] as an attractive approach to overcome the described difficulties during RCM reactions in the solid-phase bound uPA-epitope peptides.

As expected, the introduction of Mutter's pseudoproline Ser($\Psi^{Me,Me}$pro)[12] at Ser^6 in the peptide epitope yielding peptidyl resins **4**−**6** enabled the RCM reactions to proceed smoothly with excellent to good conversion rates (**4**, 81%; **5**, 84%; **6**, >95%) in all model peptides on trityl polystyrene resin (see Figure 2).[13] In the same peptides lacking the reversible backbone protection at Ser^6 either the starting material was detected or polymerization occurred.

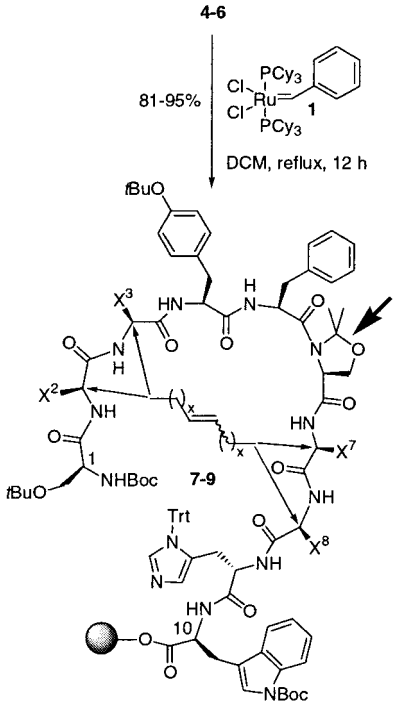

Figure 2. Series of model peptides **7**−**9** synthesized by RCM with the Grubbs catalyst **1** in refluxing CH_2Cl_2 starting from the linear olefinic and backbone protected peptides **4**−**6** on solid-phase (X^2−X^8 are either the bridging positions or the originally occurring amino acid side chains).[5] Olefinic side chain positions are Lys^3/Asn^7 (**4**) and Asn^2/Asn^7 (**5**) with $x = 1$, respectively, and Asn^2/Ile^8 with $x = 3$ (**6**), leading to hydrocarbon linker lengths of four (**4**, **5**) and eight atoms (**6**), respectively.

The *C*-terminal amino acid side chains of His(Trt) and Trp(Boc) turned out to be problematic with regard to standard peptide purification protocols and the further reduction of the generated double bond.

Mild acidic cleavage of the fully protected peptides from the resin[14] resulted in highly colored peptides **10**−**12** that could not be purified by precipitation or even by RP-HPLC.

(9) (a) Rich, D. H.; Tam, J. P. *Tetrahedron Lett.* **1977**, *9*, 749−750. (b) Goldring, W. P. D.; Hodder, A. S.; Weiler, L. *Tetrahedron Lett.* **1998**, *39*, 4955−4958. (c) Creighton, C. J.; Reitz, A. B. *Org. Lett.* **2001**, *3*, 893−895.

(10) (a) Reichwein, J. F.; Wels, B.; Kruijtzer, J. A. W.; Versluis, C.; Liskamp, R. M. J. *Angew. Chem., Int. Ed.* **1999**, *38*, 3684−3687. (b) Hirohashi, M.; Tamamura, H.; Otaka, A.; Ibuka, T.; Arakaki, R.; Nakashima, H.; Fujii, N. *Peptides*, Proceedings of the 25th European Peptide Symposium, Budapest 1998; Bajusz, S., Hudecz, F., Eds.; Akadémiai Kiadó: Budapest, 1999; pp 662−663.

(11) (a) Haack, T.; Mutter, M. *Tetrahedron Lett.* **1992**, *33*, 1589−1592. (b) Johnson, T.; Quibell, M. *Tetrahedron Lett.* **1994**, *35*, 463−466. (c) Miranda, L. P.; Meutermans, W. D. F.; Smythe, M. L.; Alewood, P. F. *J. Org. Chem.* **2000**, *65*, 5460−5468.

(12) The Fmoc-Phe-Ser($\Psi^{Me,\ Me}$pro)-OH building block was prepared as described in Wöhr, T.; Wahl, F.; Nefzi, A.; Rohwedder, B.; Sato, T.; Sun, X.; Mutter, M. *J. Am. Chem. Soc.* **1996**, *118*, 9218−9227 or purchased from NovaBiochem, Läufelfingen.

(13) Furthermore, the identical backbone protected but otherwise *C*-terminal shortened peptidyl resins lacking residues His and Trp already react at room temperature.

Testing of several commercially available hydrophilic thiol compounds lead us to a procedure for the purification of the cyclic peptides by the formation of a water-soluble ruthenium/dithiothreitol (DTT) complex and subsequent precipitation of the cyclized, fully protected peptide in water.[15]

In the following attempts to reduce the double bond in the peptide cycles **10**−**12**, the indole ring of the tryptophane residue turned out to be unstable against the reductive reaction conditions as a result of long reaction times. These findings together with the troublesome complexation properties of the *C*-terminal amino acid side chains led us to the syntheses of biologically equivalent substitutions of His[9] by Ala and Trp[10] by Phe.[16]

Finally, we synthesized a set of linear peptidyl resins consisting of the four combinations of possible cyclization positions (Asn[2]/Asn[7] **13**, Asn[2]/Ile[8] **14**, Lys[3]/Asn[7] **15**, Lys[3]/Ile[8] **16**) with racemic olefinic amino acids ($x = 2$, **a**; $x = 3$, **b**).[17] The RCM reaction proceeded smoothly, yielding the peptidyl resins **17**−**20a**,**b** (see Figure 3). To facilitate analyses of the product mixtures we tried to reduce the resulting *cis*/*trans* double bonds in the side chain protected peptides **21**−**24a**,**b** in solution as described in the literature for similar cases.[18] In the following, we observed remarkable differences in the reactivity of the cyclic peptides depending on the number of carbon atoms in the side chain bridge.

Whereas all peptide cycles **21**−**24a** could be reduced with a system of Pd/C and H_2 in DMAc within 2−3 days, this system failed in the cases of peptides **21**−**24b**. Finally we could achieve the reductions of **21**−**24b** with in situ generated diimine in NMP as solvent in a similar reaction time.[19] The reduced peptide mixtures **25**−**28a,b** were deprotected and purified by standard peptide methodology,[20] yielding the ring size library **29**−**32a**,**b** (Figure 4) as eight sets of four enantiomeric cyclic peptides.

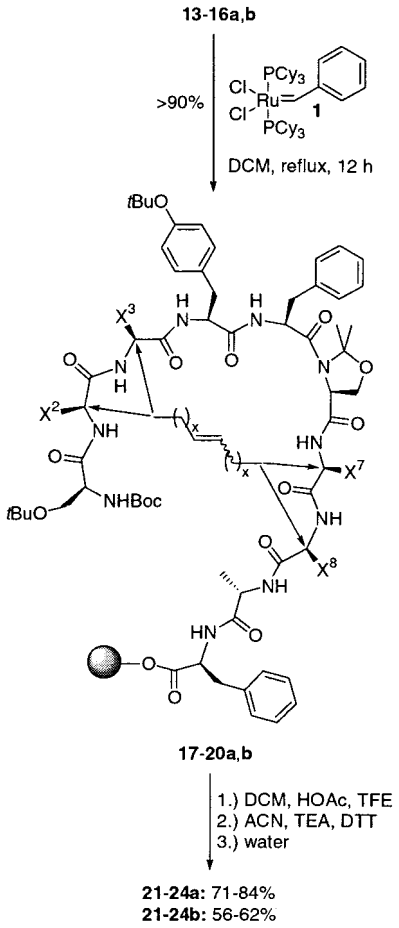

Figure 3. RCM reactions of peptidyl resins **13**−**16a,b** leading to a side chain protected ring size library containing peptidyl resins **17**−**20a,b** combining all possible cyclization positions (Asn[2]/Asn[7] **13**, Asn[2]/Ile[8] **14**, Lys[3]/Asn[7] **15**, Lys[3]/Ile[8] **16** with different lengths of the bridging sid -chains (**a**, $x = 2$, six atom bridge; **b**, $x = 3$, eight atom bridge; X^2−X^8 are either the bridging positions or the naturally occurring protected amino acid side chains).

In summary, we found that only the introduction of the reversible backbone protection Ser($\Psi^{Me,Me}$pro) enabled the side chain cyclization of 10mer peptides with unsaturated side chains of different lengths by RCM on solid phase. Investigations identified terminal butenyl and pentenyl side chains as especially suitable for the syntheses of small racemic libraries because of high conversion rates (>90%) in RCM reactions. This strategy was used for the generation of a set of cyclic peptides with ring sizes varying between 19 and 27 atoms and different stereochemical orientations at the bridging positions. Careful attention must be paid to the reduction of the resulting double bonds in the fully

(14) After the RCM reaction, the cleavage of protected peptides from the resin requires triple treatment with DCM/TFE/HOAc (8:1:1) with extended reaction times of 1 h, respectively.

(15) About 40 mg of the crude peptide was dissolved in 2 mL of ACN/TEA; 25 equiv DTT was added; the solution was incubated for 2 h, filtered, and dropped in 20 mL of water; and the precipitated peptide was collected by centrifugation, washed with water, and lyophilized from dioxane.

(16) Wilhelm, O. G.; Kessler, H.; Bürgle, M.; Potthoff, N.; Schmiedeberg, N. PCT Int. Appl. EP00/06905, 2001.

(17) Racemic olefinic amino acids were either purchased as the free amino acid (aminopentenic acid, Aldrich) and subsequently Fmoc protected or synthesized starting from diethyl acetamidomalonate and the appropriate alkenyl bromide as described in Biagini, S. C. G.; Gibson, S. E.; Keen, S. P. *J. Chem. Soc., Perkin Trans. 1* **1998**, *16*, 2485−2499. As found in peptidylresins **4** and **5** and in **14** and **16** (using enantiomerically pure allylglycine, $x = 1$, data not shown), we considered the approach with $x = 1$ as not suitable for combinatorial purposes because of a too high degree of unreacted peptide in some cases together with very low reaction rates during reduction (see further syntheses of **21**−**24a**,**b**).

(18) Ripka, A. S.; Bohacek, R. S.; Rich, D. H. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 357−360 and ref 3b.

(19) (a) Cusack, N. J.; Reese, C. B.; Risius, A. C.; Roozpeikar, B. *Tetrahedron* **1976**, *32*, 2157−2162. (b) Lacombe, P.; Castagner, B.; Gareau, Y.; Ruel, R. *Tetrahedron Lett.* **1998**, *39*, 6785−6786. (c) Reference 4. Typically 8 mg of peptide in 500 μL of NMP was added to about 30 equiv of trisylhydrazide and 1.6 equiv of DIEA (referring to trisylhydrazide) and the mixture was shaken at 50 °C for 24 h. The procedure was then repeated once for 48 h.

(20) Side chain deprotection and purification was performed as already described in ref 7 by treatment with TFA/TIPS/water (addition of 5% TFMSA to the TFA mixture mandatory for complete deprotection!), precipitation in Et_2O, and subsequent purification by RP-HPLC. Only incomplete precipitation of the deprotected peptides **29**−**32a**,**b** was achieved even by the addition of up to 40% hexane to Et_2O, which is mainly responsible for the low yields of the target molecules.

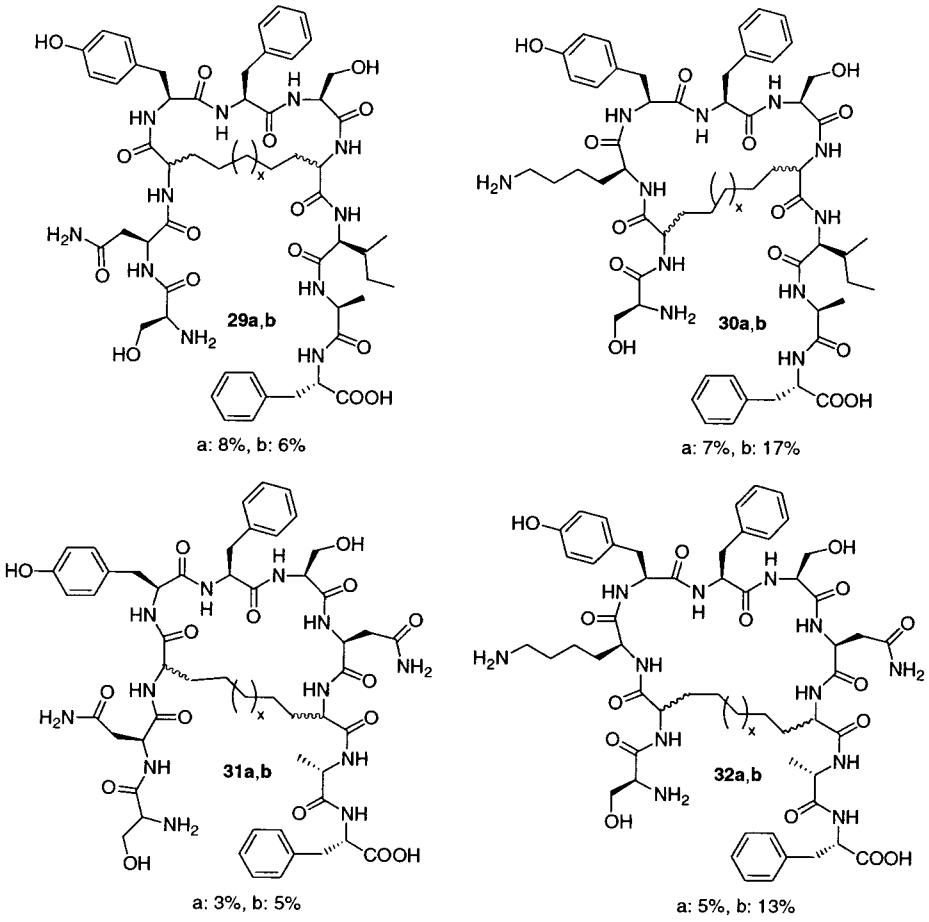

Figure 4. Reduced and deprotected set of 32 peptides **29**−**32a**,**b** (**a**, $x = 2$; **b**, $x = 4$) representing the ring size library synthesized by solid-phase RCM of pseudo-proline-protected peptides, followed by reduction and side chain deprotection. Each set consists of the approximately equimolar mixture of the four L/L, D/L, L/D and D/D peptides with ring sizes varying from 19 to 27 atoms. Yields are given for reduction, deprotection, and subsequent purification by precipitation and RP-HPLC.

protected cyclized compounds, since different ring sizes required the use of different reduction protocols.

Evaluation of the potential for fully automized combinatorial solid phase syntheses of cyclic peptide mimetic libraries is currently under investigation.

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Supporting Information Available: Characterization data for olefinic Fmoc amino acids and RCM-cyclic peptides **3**, **10**−**12**, **21**−**24a**,**b**, **25**−**28a**,**b** and **29**−**32a**,**b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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