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Conformationally Constrained Mimics of the Membrane-Proximal Domain of FcεRIα

Carsten Peters,^[a] Markus Bacher,^[a]
 Christoph L. Buenemann,^[a] Franz Kricek,^[a] Jean-
 Michel Rondeau,^[b] and Klaus Weigand^{*[a]}

The response to an allergen is initiated by the interaction of allergen-specific immunoglobulin E (IgE) with its high affinity receptor FcεRI, which is expressed on the surface of mast cells and basophils. Allergen-mediated cross-linking of FcεRI-bound IgE initiates receptor aggregation and subsequent cell activation. This results in the release of vasoactive and bronchoconstrictive mediators that trigger the clinical symptoms of type I hypersensitivity.^[1] The mouse IgG1/κ monoclonal antibody (mAb) 5H5F8 recognizes a linear peptide sequence (K¹⁷¹APREKYWL¹⁷⁹) within the membrane-proximal extracellular region of the human high affinity IgE receptor α-chain (ecFcεRIα).^[2] Antibody 5H5F8 and its Fab fragment have been shown to block IgE-mediated activation of human basophils without affecting the interaction of IgE with FcεRI. The 5H5F8 epitope or “stalk region” has thus been implicated in FcεRIα-dependent cell activation.^[3] For the design of small molecules that display similar cellular effects as 5H5F8, and which could potentially be developed into new treatments for IgE-mediated allergic conditions, we became interested in the 3D structure of K¹⁷¹APREKYWL¹⁷⁹. NMR spectroscopy studies, not surprisingly, revealed that the KAPREKYWL peptide has no distinct secondary structure in aqueous solution, and is therefore not suitable for interaction studies with potential small ligands. Thus, we considered synthetic, constrained KAPREKYWL mimics, in which the backbone and side chains adopt a similar conformation as in the native cellular environment, to be suitable tools. Crystal structures of ecFcεRIα alone and in complex with an IgE Fc fragment have been determined, but no structural information for the stalk region was provided in these studies.^[4] A crystal structure^[5] of K¹⁷¹APREKYWL¹⁷⁹ in complex with the Fab fragment of 5H5F8 was therefore used as starting point for the design of such peptide mimics; this approach assumes that this 3D structure represents a good approximation of the conformation on the cell surface. Based on analysis of this structure, we anticipated that the conformation of the nonapeptide in complex with 5H5F8 could be mimicked by introducing an alkyl- or alkenyl

bridge between Ala172 or Pro173, and Lys176. This approach offers the advantage of fine tuning by variation of the chain length.

For the synthesis of these peptide mimics we decided to apply ring-closing metathesis (RCM) chemistry. Based on the pioneering work of Grubbs et al.^[6] several examples of the synthesis of peptidic macrocycles have been published.^[7] The RCM approach has also been used for the stabilization of other preformed secondary-structure motifs, like α helices,^[8] or for the synthesis of disulfide mimics.^[9]

In this communication we describe the design, in silico evaluation and selection of peptidomimetics, their synthesis by RCM, as well as their initial evaluation by binding studies to 5H5F8.

Analysis of the X-ray structure^[5] of the Fab fragment of 5H5F8 in complex with ¹⁷¹KAPREKYWL¹⁷⁹ revealed that the peptide binds in an irregular (or “random coil”) conformation, with Arg174, Glu175, and Tyr177 buried in the antigen-binding site. It is worth noting that the side chains of Arg174 and Glu175 formed a tight intramolecular salt bridge. This salt bridge and the orientation of the side chain of Tyr177 squeezed the peptide into a shape in which the main chain dihedral angles of Arg174 and Tyr177 were within the (right-handed) alpha-helical region (approximately $\Phi = -60^\circ$, $\Psi = -50^\circ$), while all other residues adopted a more extended conformation (Figure 1, Table 1).

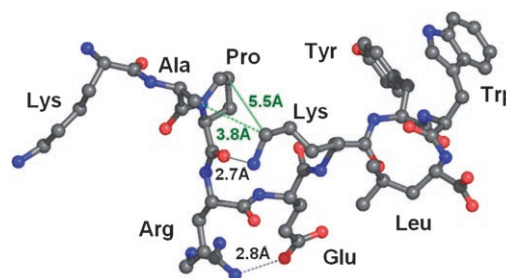


Figure 1. Structure of the KAPREKYWL peptide obtained from the X-ray structure of the cocrystal with Fab fragment of 5H5F8.

Table 1. Observed main chain dihedral angles for the bound conformation of peptide KA¹⁷²PREKYW¹⁷⁸L. The first and last amino acids are not shown because one angle is not defined at the peptide termini.

Peptide residue	Φ	Ψ
Ala172	−60.4	137.2
Pro173	−59.9	154.9
Arg174	−64.6	−44.6
Glu175	−106.4	120.2
Lys176	−94.3	139.4
Tyr177	−57.5	−49.1
Trp178	−123.6	141.1

Previous epitope mapping^[3] of the KAPREKYWL peptide demonstrated that Pro173 is not directly involved in binding to 5H5F8, but enhances affinity, whereas at position 176 all amino acids except cysteine are tolerated. We therefore chose these two residues as anchor points for attaching the alkenyl handles required for the planned RCM chemistry. A representa-

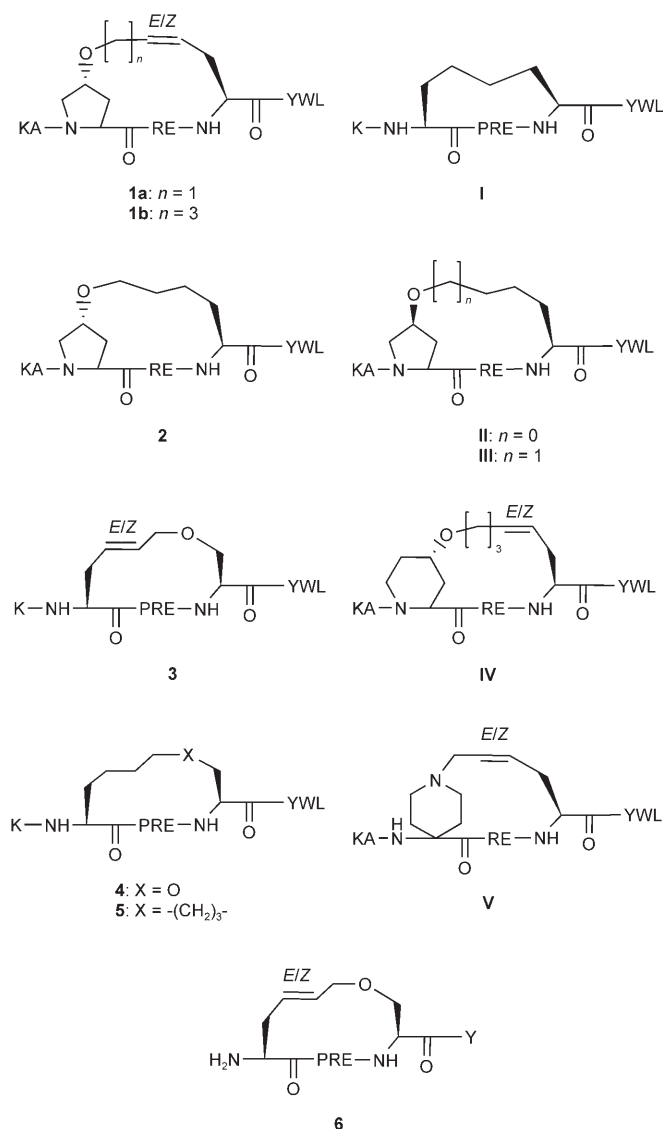
[a] Dr. C. Peters, Dr. M. Bacher, Dr. C. L. Buenemann, Dr. F. Kricek, Dr. K. Weigand
 Novartis Institutes for BioMedical Research
 Brunner Strasse 59, 1235 Vienna (Austria)
 Fax: (+43) 1-80166-354
 E-mail: klaus.weigand@novartis.com

[b] Dr. J.-M. Rondeau
 Novartis Institutes for BioMedical Research
 Lichtstrasse 35, 4056 Basel (Switzerland)

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tive subset of the target molecules we chose for computational and experimental proof of our approach is depicted in Scheme 1. Replacement of Lys176 by (*S*)-allylglycine and Pro173 by *O*-alkenyl-(2*S*)-hydroxyproline, with variation of the stereochemistry and length of the *O*-alkenyl chain at position 4, led to macrocycles **1**, **2**, **II**, and **III** after RCM and hydrogenation where appropriate. Similarly, the impact of variations of the ring size and backbone conformation were evaluated by formal replacement of Pro173 with (2*S*,4*S*)-4-allyloxy-piperidine-2-carboxylic acid and 1-allyl-4-amino-piperidine-4-carboxylic acid (which led to RCM products **IV** and **V**). In another set of bridged peptides, Ala172 was substituted by (*S*)-allyl-glycine, and Lys176 was replaced by *O*-allyl-(*S*)-serine to give **3** and **4**, respectively; Lys176 was also substituted by (*S*)-heptenyl-glycine to give **5**, or (*S*)-allyl-glycine to obtain **I** (Scheme 1).

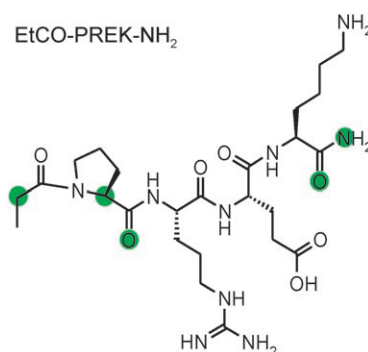
To prioritize the synthetic-chemistry efforts, the target molecules were ranked by using computational tools. Modeling experiments by using QXP^[10] were performed in which candidate



Scheme 1. The bridged KAPREKYWL mimics discussed in the text. Molecules with roman numerals were evaluated by molecular modeling only.

molecules were superposed onto KAPREKYWL. The 3D coordinates of the KAPREKYWL template were taken from the cocrystal with the Fab fragment of the 5H5F8 mAb, and these coordinates were kept fixed in the experiment. The experimental setup was chosen in order to reduce the complexity of the computational problem (Scheme 2). Two examples of the superposition results are shown in Figure 2. Prioritization of mimics was based on an empirically derived overall score that considered conformational-strain energy, violation of distance constraints, and fit to the 5H5F8 mAb binding site, based on the KAPREKYWL cocrystal structure (Table 2, and legend).

Compounds **3**, **4**, and **5** scored highest in the triaging process and were therefore selected for chemical synthesis. For probing the computational results, **1a/b** and **2** were also synthesized. Peptide **6**, which can be traced back to **3** by deletion



Scheme 2. Setup of superposition experiments. To reduce noise from insufficient sampling during superposition, those parts that were common to all molecules were removed; this led to EtCO-PREK-NH₂ and its mimics. Both ends of the molecules needed to be positioned and orientated such that reading the removed parts (N-terminal NH₂-CONH₂, and C-terminal YWL-COOH) would result in a good fit to the full-length template of KAPREKYWL. At least two constraints on either side of the modeled molecule were needed for correct positioning of the ends, and for both ends' correct orientation. Distance constraints of 0 Å for five backbone atom pairs (green) were found sufficient, and TFit^[10] was run with 2000 cycles of optimization.

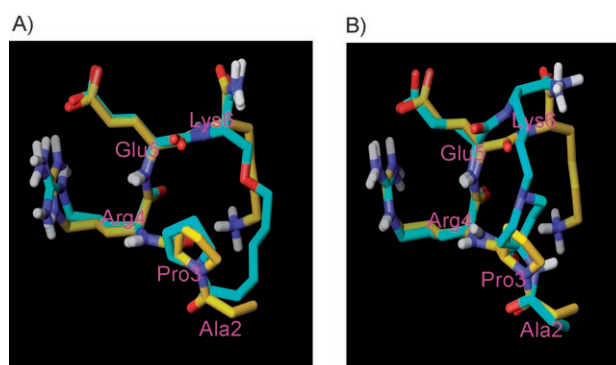


Figure 2. Two examples of superposition results. Carbon atoms of the template and of the candidate molecule are shown in orange and cyan, respectively. A) Good fit of compound **4**. Atoms involved in distance constraints superposed nicely (constraint energy 3.4 kJ mol⁻¹). Backbones of the two molecules were well aligned (root mean square deviation (RMSD) 0.27 Å, maximum distance 0.66 Å). Conformational strain of the candidate molecule was low (5.4 kJ mol⁻¹). B) Poor fit of compound **V** (*E* isomer). There were significant violations of the distance constraints (constraint energy 13.8 kJ mol⁻¹). The backbone atom positions deviated significantly from the template (RMSD 1.00 Å), in particular for the C- α atom of Lys6 (2.59 Å). Conformational strain of the candidate molecule was high (21.1 kJ mol⁻¹).

Table 2. Overall score and ranking of candidate compounds.^[a]

Compound	$QXP_{\text{constraint}}$ [kJ mol ⁻¹]	$QXP_{\text{conformation}}$ [kJ mol ⁻¹]	$Z_{\text{superposition}}$	Contact			Z_{fit}	Z_{overall}
				A	B	C		
KAPREKYWL	1.0	2.8	-0.99	118	1	0	-1.34	-1.7
5	1.4	0.9	-1.07	132	13	4	-0.58	-1.4
4	3.4	5.4	-0.72	121	11	4	-0.62	-1.0
(Z)- 3	4.0	7.2	-0.59	126	11	5	-0.56	-0.9
(E)- 3	2.9	7.6	-0.63	134	12	10	-0.14	-0.7
II	1.3	18.7	-0.12	120	4	2	-1.06	-0.7
III	6.5	14.3	-0.08	89	4	1	-1.02	-0.6
(Z)- 1 a	6.1	14.8	-0.07	91	5	5	-0.66	-0.4
(E)- 1 a	3.8	14.9	-0.19	115	11	11	-0.02	-0.2
2	2.5	11.3	-0.45	136	17	19	0.80	-0.1
(Z)- 1 b	2.0	11.0	-0.50	147	23	21	1.16	0.1
(E)- 1 b	3.9	10.5	-0.42	153	21	29	1.71	0.4
(Z)- V	22.7	15.3	0.85	119	22	18	0.99	1.3
(E)- I	28.2	25.0	1.66	125	16	5	-0.35	1.5
(E)- V	13.8	21.1	0.68	147	26	28	1.86	1.6
(Z)- I	33.7	37.6	2.64	117	19	5	-0.19	2.5

[a] A scoring system was defined to evaluate the quality of the superposition of truncated candidate molecules (Scheme 2) on the corresponding sequence derived from the 5H5F8-KAPREKYWL X-ray structure. $QXP_{\text{constraint}}$ is the constraint energy that reflects deviations from constraints as defined in Scheme 2. $QXP_{\text{conformation}}$ is defined as the conformational strain energy of the superposed molecules. The sum of both terms is normalized by the Z-score method to give $Z_{\text{superposition}}$, which reflects the quality of the superposition. To consider how well the molecules fit to the binding site of mAb 5H5F8, the number of favorable van der Waals contacts (contact A), slight van der Waals clashes (contact B), or heavy van der Waals clashes (contact C) between the designed linker and 5H5F8 were derived by using Maestro's^[14] measurement tool at default settings. To mirror the overall quality of a molecule's fit to the receptor, the term "fit" was defined as $\text{fit} = -0.05 \times \text{contact A} + 0.5 \times \text{contact B} + 1.0 \times \text{contact C}$. The Z_{overall} was defined as $Z_{\text{overall}} = Z_{\text{superpos}} + 0.5 \times Z_{\text{fit}}$, which introduced an arbitrary weighting factor of 0.5 to take into consideration the uncertainty associated with contact analysis as molecules were not optimized for fitting to 5H5F8. Low Z_{overall} values are equivalent to a better result. Because all candidate molecules were truncated to their corresponding EtCO-PREK-NH₂ core for the in silico assessment, compound **6** was indistinguishable from **3** in this analysis. However, the weaker binding of **6** (in comparison to **3**) was expected based on the known contribution of flanking residues to binding (see text).

of the amino-terminal lysine and carboxy-terminal tryptophanyl-leucine, was chosen for synthesis in order to investigate the influence of adjacent amino-acid residues on binding to the antibody.

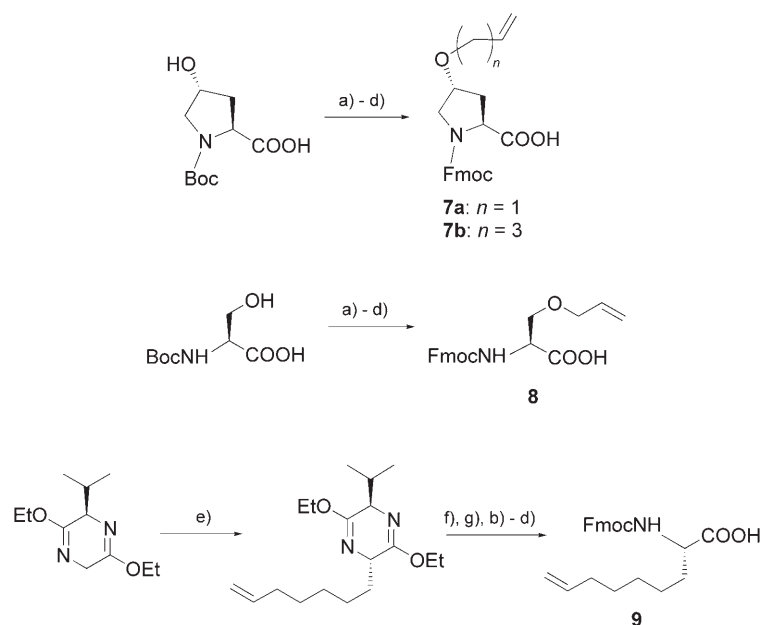
The key step in the synthesis of the bridged peptides was the introduction of the alkene linkage by RCM. Most of the published examples have applied allylglycine or homoallylglycine as precursors for RCM, but our target molecules required *O*-alkenylated hydroxyproline **7a/b** and *O*-allylserine (**8**) as building blocks for the metathesis reaction.

Compounds **7** and **8** were readily obtained by a four-step reaction sequence. The *N*-Boc-protected amino acids were treated with excess sodium hydride and an alkenyl bromide to give the corresponding bis-alkenylated derivatives (Scheme 3). The ester groups were then saponified by treatment with aqueous base. Finally, the *N*-terminal Boc group was replaced by an Fmoc group in order to make these building blocks suitable for Fmoc solid phase peptide synthesis. The corresponding alkenylated glycine-derivative **9** was synthesized by using the bislactim ether protocol.^[11]

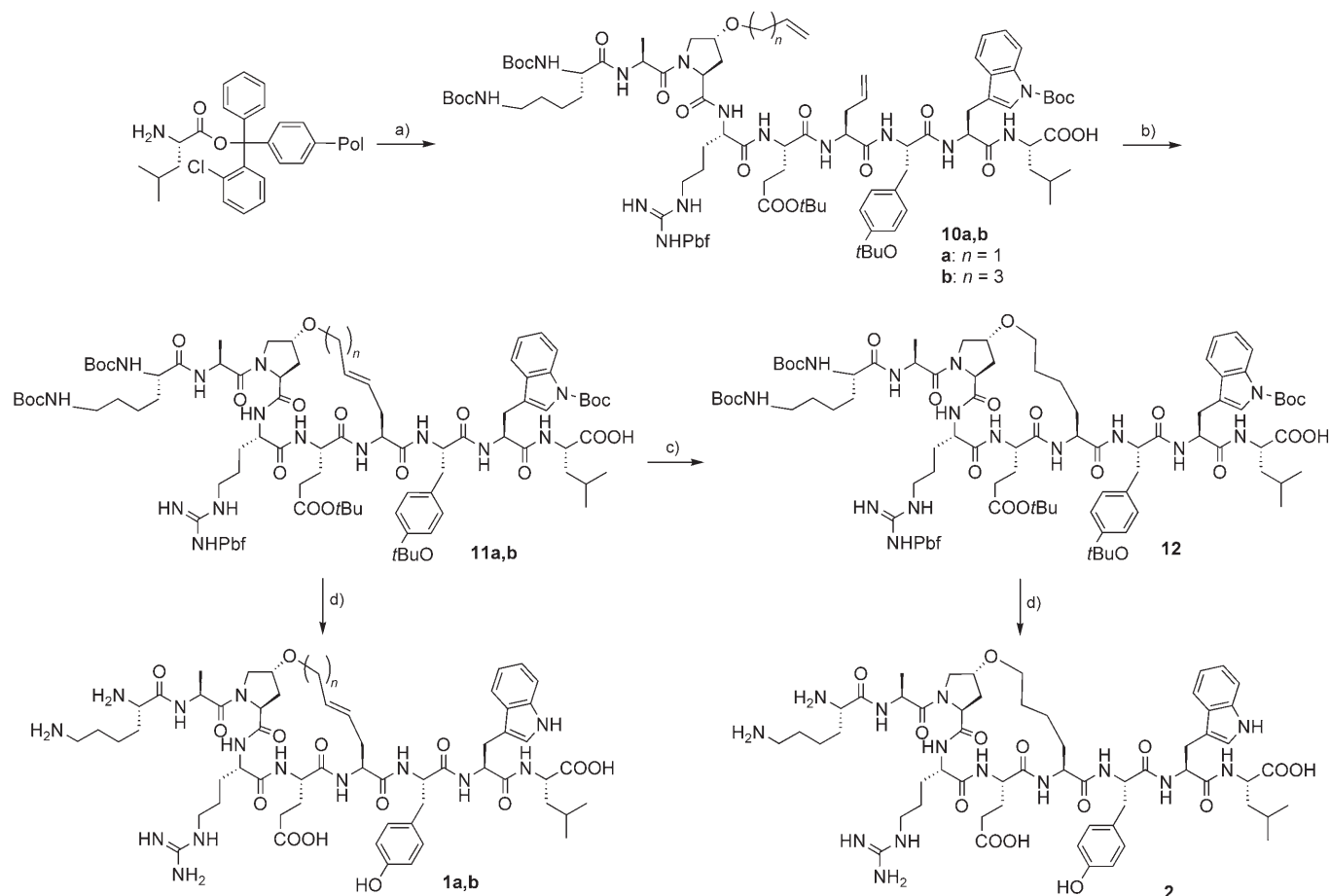
Scheme 4 shows the synthesis of **1** and **2**; this was initiated with the generation of the acyclic peptides **10a** and **b**, which incorporated the building blocks

7a and **7b** under standard conditions on 2-chlorotrityl resin. The fully protected peptide acids were obtained by cleavage from the resin under mild acidic conditions and purified by preparative HPLC, which gave the open-chain compounds **10a** and **b** in high yield and purity. The critical RCM was performed by using Grubbs' catalysts in dichloromethane at a concentration of 5 mM.

There was no obvious preference for a certain type of catalyst. For the allyl-containing peptide **11a**, the yields ranged between 18% (second generation Grubbs' catalyst) and 36% (second generation Hoveyda-Grubbs' catalyst). For the pentenyl-ether **11b**, the first generation Grubbs' catalyst (44%) was found to be superior to the Hoveyda-Grubbs' catalyst (23%). Peptide **11a** was formed with an *E/Z* ratio between 2:1 to 1:1, as determined by NMR spectroscopy, whereas RCM treatment of



Scheme 3. Synthesis of building blocks **7–9** used for the generation of RCM precursors. Reagents and conditions: a) NaH, allyl bromide (for **7a**, **8**) or 5-bromopent-1-ene (for **7b**), DMF, room temperature; b) LiOH; THF/MeOH/water, room temperature; c) CF₃COOH/CH₂Cl₂, room temperature; d) Fmoc-OSu, iPr₂NEt, THF, 0 °C to room temperature; e) *n*BuLi, 7-bromohept-1-ene, -78 °C to room temperature; f) HCl (1 N), room temperature; g) Boc₂O, dioxane, room temperature followed by chromatographic separation from Boc-Val-OEt.



Scheme 4. Synthetic approach towards constrained KAPREKYWL mimics with Pro173 replacements. Reagents and conditions: a) Fmoc-AA-OH, HBTU, HOBT, $i\text{Pr}_3\text{NEt}$, *N*-methyl pyrrolidone, room temperature, 1 h; b) $[\text{RuCl}_2(\text{PCy}_3)_2(=\text{CHPh})]$, CH_2Cl_2 , reflux; c) H_2 , Pd/C, EtOH, room temperature; d) $\text{CF}_3\text{COOH}/(i\text{Pr}_3\text{SiH}/\text{H}_2\text{O}$, 95:2.5:2.5 (v/v/v), room temperature.

peptide **10b** with first generation Grubbs' catalyst gave **11b** with an *E/Z* ratio of up to 20:1. Attempts to separate the *E/Z* isomers by chromatography were not successful.

The macrocycles **11a** and **11b** were then deprotected by treatment with acid, which led to alkene-bridged peptides **1a** and **1b**. By pursuing a similar strategy with the incorporation of appropriate building blocks, the macrocycles **3–6** were obtained. The RCM reaction towards cyclic peptide **3** was performed in the presence of first generation Grubbs' catalyst; this delivered the fully protected peptide in up to 77% yield as a 4:1 *E/Z* mixture. The metathesis reaction towards peptide **5** delivered the fully protected macrocycle with a yield of 51% (first generation Grubbs' catalyst) and an *E/Z* ratio of 2:1. Hydrogenation of the double bond and subsequent removal of the protecting groups gave **5**. Hydrogenation of peptide **11a** yielded **12**, which was converted to peptide **2** by removal of the protecting groups. The formation of the macrocyclic precursor of peptide **6** was achieved with a yield of 27% by using first generation Grubbs' catalyst.

For experimental validation of the predictions obtained from the modeling studies, the deprotected alkene- and alkane-bridged peptides **1–6** were tested for their binding to 5H5F8

by using surface plasmon resonance (SPR). To this end, a Biacore chip coated with mAb 5H5F8 was used. Solutions of peptides **1–6** were applied to this chip and the "on-" and "off" rates were determined;^[12] peptide KAPREKYWL was used as control (Table 2).

Peptides **3** and **4** exhibited strong binding to the antibody; this was comparable to the unconstrained peptide. The off rates of both rigidified molecules were in the same range, but slightly lower compared to the linear KAPREKYWL peptide. It is well established that conformationally constrained macrocyclic compounds can bind to their receptor with higher affinity than their linear counterparts, presumably because of a more favorable entropic contribution to binding.^[13] The increased affinity of **4** compared to **3** could be due to the higher flexibility of the saturated bridge; this suggests a requirement for a conformational change of the linker region upon binding to the antibody (Table 3).

The K_D value of the truncated peptide **6** was only about two-fold higher than that of the full-length mimic **3**, and it showed higher association and dissociation rates. This result is consistent with epitope-mapping data, which demonstrate that motif $^{173}\text{PREK}^{176}$ provides the major contribution to the binding

Table 3. Results of binding experiments of constrained KAPREKYWL mimics to immobilized monoclonal antibody 5H5F8 by using surface plasmon resonance.

Mimic	K_D [nM]	k_a [s^{-1}]	k_d [s^{-1}]
KAPREKYWL	3.94 ± 0.7	$1.47 \pm 0.6 \times 10^6$	$5.79 \pm 0.7 \times 10^{-3}$
1 a/b	no binding	–	–
2	no binding	–	–
3	5.56 ± 0.4	$6.06 \pm 1.2 \times 10^5$	$3.37 \pm 0.4 \times 10^{-3}$
4	1.10 ± 0.1	$3.35 \pm 0.3 \times 10^6$	$3.57 \pm 0.1 \times 10^{-3}$
5	no binding	–	–
6	11.6 ± 2.8	$1.72 \pm 1.9 \times 10^6$	$1.99 \pm 0.6 \times 10^{-2}$

of KAPREKYWL to 5H5F8.^[3] Compounds **1 a/b**, **2**, and **5** did not show any detectable binding to 5H5F8. For **1 a/b**, **2** this was in agreement with the modeling results, while **5** was predicted to be a good binder. In spite of this single false positive result, the in silico compound prioritization proved to be a valuable tool for guiding the chemistry efforts.

In summary, a successful approach for the synthesis of conformationally constrained analogues of the KAPREKYWL fragment of FcεRIα is described. Based on results obtained from molecular-modeling studies, constrained analogues of KAPREKYWL were selected for synthesis. Ring closing metathesis proved to be a valuable tool for the generation of the peptidic macrocycles. Results from antibody-binding assays indicated that several of the macrocyclic mimics had an affinity for the mAb that was comparable to KAPREKYWL. These results correlated well with the predictions from the molecular-modeling studies. With peptides **3** and **4** we have compounds that mimic the conformation of KAPREKYWL within its native environment, that is, as “stalk region” of full-length FcεRIα. This opens new avenues for the identification of low-molecular weight ligands that bind to the membrane-proximal region of the α chain of FcεRI.

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Keywords: antibodies • computational chemistry • constrained peptides • peptidomimetics • ring-closing metathesis

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