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Synthesis and Structure−Activity Relationships of Dimeric Peptide Antagonists of the Human Immunoglobulin G−Human Neonatal Fc Receptor (IgG−FcRn) Interaction

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The neonatal Fc receptor, FcRn, regulates the half-life of IgG in vivo and may be a target in the treatment of autoimmune disease. Monomeric peptide antagonists of the human IgG−human FcRn interaction were dimerized using three different synthetic methodologies: thiol/alkyl halide coupling of unprotected peptides, reductive alkylation of unprotected peptides, and on-resin amide bond formation with protected peptides. It was found that dimerization of monomeric peptides increased the in vitro activity of the peptide monomers more than 200-fold. Human IgG catabolism experiments in human FcRn transgenic mice were used to assess the in vivo activity of peptide dimers that possessed different linkers, cyclizations, and affinities for FcRn. Overall, it was found that the linker joining two monomeric peptides had only a minor effect on the in vitro potency but that in vitro potency was predictive of in vivo activity.

Introduction

The neonatal Fc receptor, FcRn,[a] is a 42 kDa heterodimeric MHC class-I-like protein consisting of a heavy chain in complex with β2-microglobulin (β2m).[1] FcRn is expressed across many different tissues and cell types and is the receptor responsible for providing both IgG and albumin molecules with their characteristically long half-life in vivo. For example, IgG1 molecules have a typical serum half-life of 3 weeks in humans, while other proteins of similar size may only have a half-life on the order of days or less. The currently accepted mechanism of action is as follows: after the nonspecific uptake of IgG into cells' acidic endosomes by pinocytosis, FcRn binds to IgG at pH 6 and shuttles the protein back to the cell surface, where IgG is released from FcRn because of lack of binding at pH 7.4. In the process, FcRn diverts IgG from the degradative lysosomal pathway.[2,3]

The fact that FcRn can modulate the half-life of IgG molecules has led to the investigation of this receptor as a therapeutic target in autoimmune disease.[4,5] By use of FcRn knockout mice, it has been shown that FcRn is implicated in disease models for rheumatoid arthritis[6] and the skin blistering diseases pemphigus[7] and epidermolysis bullosa acquisita.[8] A monoclonal antibody against FcRn has shown efficacy in experimental autoimmune myasthenia gravis in rats.[9] And last, an Fc domain of IgG engineered for higher affinity to FcRn has also shown efficacy in experimental arthritis in mice.[10]

We recently described a family of peptides that binds to human FcRn (hFcRn) and blocks the binding of human IgG (hIgG) both in vitro and in vivo.[11] The peptides contained a disulfide loop as well as the consensus sequence Gly-His-Phe-Gly-Gly-X-Tyr, where X is preferably a hydrophobic amino acid. Extensive structure−activity studies were performed on one of the peptides from the consensus sequence family, and it was found that the addition of four methyl groups to the sequence increased the in vitro activity of the peptide monomer by 50- to 100-fold.[12] Inhibition of FcRn function in nonhuman primates with the chemically optimized peptide homodimer **1** (SYN1436) led to a dramatic reduction in IgG concentrations by inhibiting the natural IgG recycling pathway.[11] Critical to the in vivo efficacy of peptide **1** was the homodimerization of one of the most potent monomers, peptide **2** (SYN1327). Herein, we describe three different synthetic strategies used to generate various peptide dimers, as well as their structure−activity relationships using both in vitro and in vivo analyses.

Results

Synthesis of Peptide Dimers via Thiol/Alkyl Halide Chemistry. During the SAR study of monomeric peptide **3** (Table 1), it was hypothesized that a dimeric form of the peptide might be more potent inhibitor of FcRn (vide infra). Since it was found that certain lactam-cyclized monomers were more potent than the Cys-Cys cyclized monomers,[12] they were chosen as first candidates for dimerization. An additional benefit of the lactam peptides was that there would be no complex issues of disulfide shuffling. A chemoselective homodimerization strategy that included thiol/alkyl halide chemistry was used to generate lactam-cyclized homodimers. This was accomplished by reacting bis-thiol linker molecules with bromoacetylated lactam-cyclized peptides (Figure 1). This methodology was applied to peptide **14**, the Asp-Lys lactam derivative of the cysteine-cyclized peptide **3**. To generate the linker molecules, *N*,*N*-bis(*N*′-Fmoc-3-aminopropyl)glycine (BAPG) was coupled to 2-chlorotrityl resin preloaded with Fmoc-Gly-OH. To evaluate the effect of

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[a] Abbreviations: BAPG, *N*,*N*-bis(*N*′-Fmoc-3-aminopropyl)glycine; DIEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; ELISA, enzyme linked immunosorbent assay; FcRn, neonatal Fc receptor; LCMS, liquid chromatography−mass spectrometry; MHC, major histocompatibility complex; NMeLeu, *N*-methylleucine; Pen, L-penicillamine; PyBOP, benzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate; Sar, sarcosine; SATP, *N*-succinimidyl-*S*-acetylthioacetate; SPR, surface plasmon resonance; TFA, trifluoroacetic acid.

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Table 1. In Vitro Activity of Monomeric Peptides

peptide	sequence[a]	IC_{50} IgG competition ELISA assay, nM	K_d for hFcRn at pH 6 surface plasmon resonance, nM	K_d for hFcRn at pH 7.4 surface plasmon resonance, nM
3	QRFCTGHFGGLYPCNGP	40000 ± 15000 (n = 4)	5300 (n = 2)	37000 (n = 2)
10	RFCTGHFGGLYPC	37000 ± 44000 (n = 3)	5900 (n = 2)	nd
11	RF-**Pen**-TGHF-**valerolactam-NMeLeu**-YPC	430 ± 150 (n = 7)	43 ± 12 (n = 3)	260 ± 40 (n = 3)
12	RF-**Pen-NMeAla**-GHF-**X-NMeLeu**-YPC	1100 ± 30 (n = 3)	150 ± 17 (n = 3)	950 ± 580 (n = 3)
2	RF-**Pen**-TGHFG-**Sar-NMeLeu**-YPC	520 ± 260 (n = 22)	31 ± 4 (n = 3)	170 ± 20 (n = 3)
13	RF-**Pen**-TG-**4GuPhe**-G-**Sar-NMeLeu**-YPC	1000 ± 650 (n = 4)	62 ± 10 (n = 3)	74 ± 2 (n = 3)
14	QRF-**Asp**-TGHFGGLYP-**Lys**-NGP	22000 ± 19000 (n = 3)	2600 ± 1300 (n = 3)	18000 (n = 2)
15	RF-**Asp**-TGHFG-**Sar-NMeLeu**-YP-**Lys**	1900 ± 1400 (n = 3)	140 ± 30 (n = 5)	930 ± 150 (n = 3)

[a] All peptides are acetylated at the N-terminus and amidated at the C-terminus. All peptides are cyclic either through disulfide bonds (Cys−Cys, Pen−Cys) or through amide bonds (Asp−Lys). "Pen" denotes a L-penicillamine amino acid. "Sar" denotes sarcosine. "NMeLeu" denotes a *N*-methylleucine amino acid. "4GuPhe" denotes a 4-guanylphenylalanine amino acid. "X" denotes the valerolactam dipeptide (*R*)-3-amino-2-oxo-1-piperidineacetic acid.

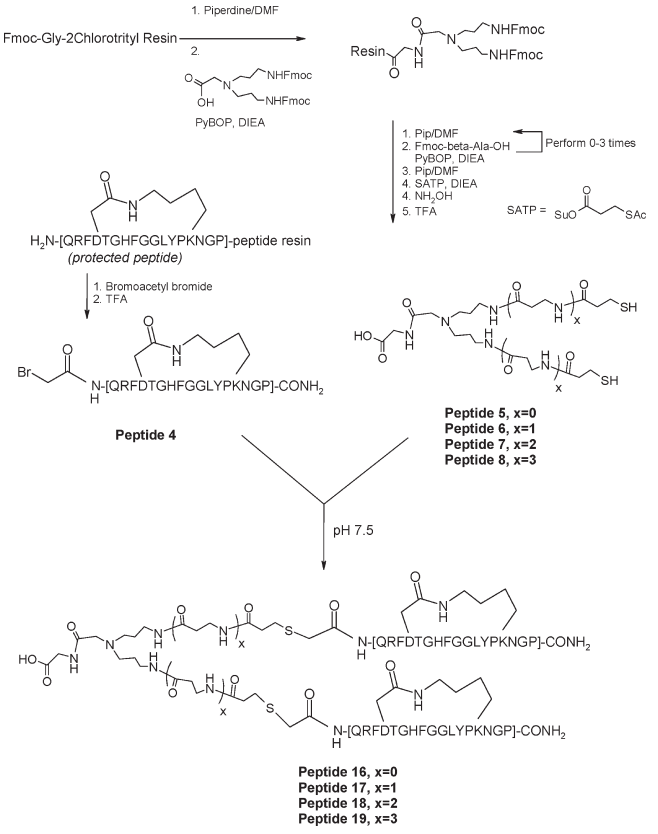


Figure 1. Synthetic scheme for generating peptide dimers using thiol/alkyl halide chemistry and bis-thiol linkers.

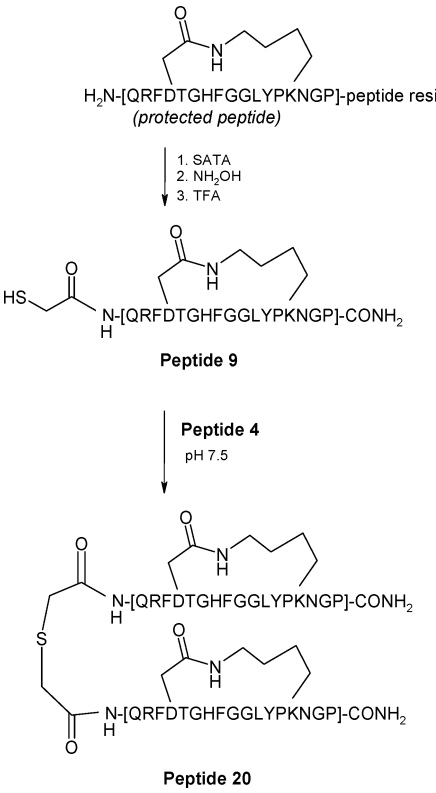


Figure 2. Synthetic scheme for generating peptide dimer **20** using thiol/alkyl halide chemistry.

linker length, zero to three β-alanine residues were incorporated into the linkers. The thiol moiety was added to the linkers by coupling the peptide resin with the protected thiol *N*-succinimidyl-*S*-acetylthioacetate (SATP) which, after treatment with hydroxylamine, generated the free thiol group. Cleavage of the linkers from the resin with TFA afforded the bis-thiol linkers with varying linker lengths. The reactive peptide was generated by reacting the lactam-cyclized peptide resin[12] with bromoacetyl bromide, followed by TFA cleavage to afford the electrophilic peptide **4**. Reaction of the bis-thiol linkers **5**−**8** with peptide **4** at pH 7.5 afforded the four lactam-cyclized peptide dimers **16**−**19** with varying distances between the peptides.

A second strategy was used to generate peptide dimers using thiol/alkyl halide chemistry by synthesizing two separate peptides: one peptide with a N-terminal thiol group and another peptide with a bromoacetyl moiety as above. A thiol group was incorporated onto the N-terminus of the peptide by reacting the crude lactam-cyclized peptide resin with SATP and hydroxylamine as above. Subsequent cleavage of the peptide from the resin afforded nucleophilic peptide **9**. Reaction of the unprotected peptides **9** and **4** at pH 7.5 afforded the dimeric peptide **20** (Figure 2).

Attempts to use the thiol/alkyl halide linkage methods with disulfide-cyclized peptides were successful but resulted in misfolded impurities due to disulfide shuffling caused by the presence of the thiol linkers (data not shown).

Synthesis of Peptide Dimers via Reductive Alkylation. An alternative approach was thus sought to dimerize peptides that contained disulfide bonds in their structures. Since substitution of the first cysteine in the sequence to L-penicillamine (Pen) provided a dramatic boost to in vitro activity,[12] it was advantageous to develop a method that was more compatible with disulfide bonds. The chosen method involved

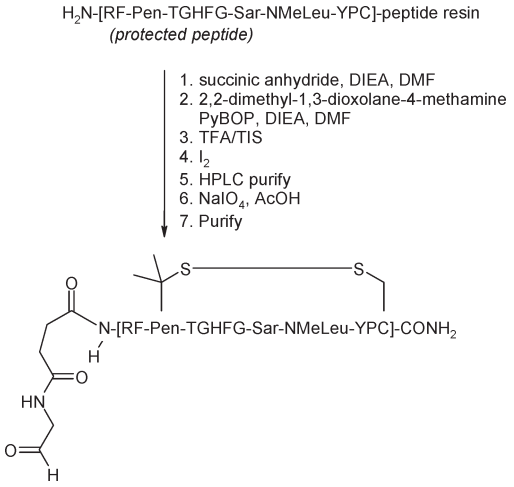


Figure 3. Representative synthesis of a peptide aldehyde that includes an internal disulfide bond.

the reductive alkylation of a peptide aldehyde with a second amine-containing peptide. Peptide aldehydes were synthesized either at the N-terminus or at the C-terminus. To generate N-terminal aldehydes, the free amino terminus of the peptide resin was coupled with 2,2-dimethyl-1,3-dioxolane-4-methamine. Subsequent cleavage of the peptides from the resin generated an unprotected diol. At this point in the synthesis, the disulfide bridge within the peptide monomer was formed using iodine and the peptide was purified using HPLC. Brief treatment of the purified diol-containing peptide with sodium periodate in acetic acid/water afforded the aldehyde-containing disulfide-bridged peptide monomer (Figure 3). The C-terminal aldehydes were synthesized similarly but by using a diol coupled to a trityl resin followed by standard peptide synthesis protocols and two oxidation steps.

To generate the amine-containing peptides, sarcosine was incorporated onto the N-terminus of the peptide following standard protocols. The use of sarcosine eliminated the possibility for two alkylations onto a primary N-terminal amine. C-Terminal amines were generated using a trityl resin derivatized with ethylenediamine. Following standard peptide synthesis protocols, cleavage from the resin resulted in a C-terminal ethylamine peptide.

This peptide aldehyde was reacted with an amine-containing peptide in the presence of sodium cyanoborohydride in 2% acetic acid/DMF for 1 h and resulted in a chemoselective reaction to generate the peptide homodimer (Figure 4). One major advantage of this approach is the fact that the disulfide bonds are formed within each peptide monomer prior to dimerization. Since no disulfide shuffling occurs during the reaction, this method ensures that the proper disulfide folding patterns remain intact. The synthesis of peptides with both N- and C-terminal aldehydes and amines allowed for the linkage of monomeric FcRn-binding peptides in different orientations using reductive alkylation chemistry. Peptide orientations included N-terminal-to-N-terminal (peptides **21**−**27**), N-to-C (peptide **35**), and C-to-C (peptide **36**).

The drawbacks of this approach are the multistep process to generate the aldehyde and the requirement to synthesize two separate peptides with complementary reactivity. However, the ability to rapidly generate peptide homo- and heterodimers is of high utility.

Synthesis of Peptide Dimers via On-Resin Dimerization. The third approach and most direct approach to chemically

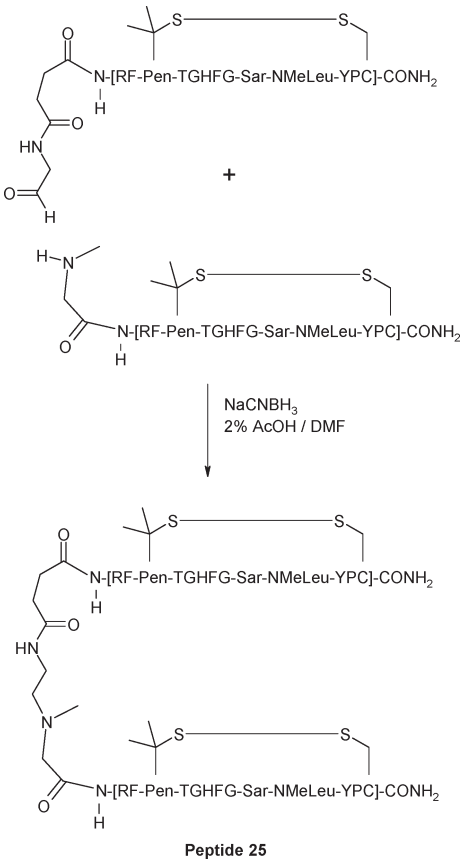


Figure 4. Representative synthesis of a peptide dimer (**25**) using reductive alkylation methodology.

dimerize the peptides was to do so directly on the peptide resin. To generate a N-to-N dimer,[13] the free N-terminus of the peptide resin was reacted with 0.5 equiv of a diacid, such as succinic acid, in the presence of a suitable coupling agent such as PyBOP and base such as DIEA (Figure 5). In this reaction, adjacent peptides on the resin bead are linked on-resin by the diacid linker. Subsequent cleavage of the peptide from the resin afforded the crude peptide. Disulfide oxidation in the presence of aqueous DMSO at pH 7.5 generated the correct intrapeptide disulfide bonds, which were verified by trypsin digestion and LCMS analysis. We found that this method was the most simple and effective way to generate the peptide dimers. This methodology was used with various diacids and generated peptides **28**−**34**.

In Vitro Activity of Peptide Dimers. The in vitro activity of the peptides was assessed using a FcRn−IgG competition assay as described previously.[11] Briefly, various concentrations of anti-FcRn peptide analogues each mixed with 3 nM human IgG were incubated with soluble human FcRn-coated plates. The concentration required to inhibit 50% of the human IgG−FcRn interaction was designated the IC_{50} value.

Over the course of studying all of the dimeric analogues, a trend was observed where the effect of dimerization on in vitro potency was more pronounced as the potency of the monomer itself was enhanced. For example, modest improvements in activity over that of the monomer (approximately 10- to 40-fold) were observed by dimerizing the relatively weak monomeric peptides **14** (into dimers **16**−**19**, Table 2) and **10** (dimer **21**, Table 3). In contrast, more dramatic improvements in activity were observed (up to 330-fold) by dimerizing the more potent peptide **2** and related peptides

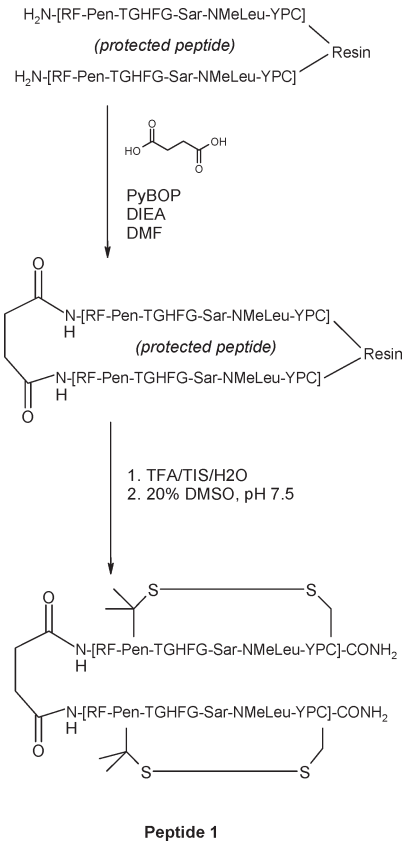


Figure 5. Representative synthesis of a peptide dimer (peptide **1**) using the on-resin dimerization method.

that contained the Pen, Sar, and NMeLeu substitutions (Tables 3 and 4).[12]

It was originally hypothesized that the length of the linker moiety bridging the two peptide monomers would have a large impact on the activity of the dimers. In general, however, this was not the case. For example, for the two dimers that were synthesized by reductive alkylation, peptide **27** with a seven-atom linker bridging the two N-termini of peptide **2** has equivalent potency (1.6 ± 0.46 nM) as peptide **26** (2.4 ± 1.1 nM) which possesses a 22-atom linker, where both of these linkers comprise mainly amide bonds (Table 4). Furthermore, peptide **1** with a four-atom succinyl linker bridging the N-termini of peptide **2** is also equipotent (2.4 ± 1.4 nM). Linker length was also studied in the context of the lactam dimers which were generated by thiol/alkyl halide chemistry. Again, the linker length did not greatly influence potency, as peptide dimers **16**−**19** with increasing linker lengths using β-alanine residues all possessed similar in vitro activities (∼1−2 μM, Table 2). We therefore conclude that linker length is not a general determinant of potency.

The composition of the linker moiety, however, appears to have a minor effect on the in vitro activity of the peptide dimers. For example, peptide dimer **30** is a N-to-N-linked dimer of peptide **2** and possesses a 12-atom linker with two central *N*-methylamide groups. Its activity in the IgG competition ELISA (5.2 ± 1.3 nM) is ∼3-fold weaker than the similar seven-atom amide-linked peptide **27** (1.6 ± 0.46 nM, Table 4). These data suggest that the flexibility, hydrophobicity, and/or hydrogen bonding capability of the linker has a minor effect on in vitro potency, although no clear trend emerged from the analogues studied.

The majority of the peptides synthesized were dimerized at their N-termini. However, peptides were also synthesized with a bridging linker at the C-termini, as well as with an N-to-C bridge, to study the effect of this change on in vitro activity (peptides **35** and **36**, Table 4). It was found that peptide dimers in an N-to-C (peptide **35**, 3.9 ± 2.4 nM) or C-to-C (peptide **36**, 4.2 ± 0.6 nM) orientation could exhibit similar activities to those peptide dimers bridged at their N-termini (generally 2−5 nM). These data suggest that the N- and C-termini are in proximity to one another and that neither terminus is near the site of receptor binding. The fact that either terminus could be used as a site of dimerization is consistent with the SAR data on the monomeric peptides which indicate that amino acids can be appended onto the ends of either the N- or C-terminus of the core peptide sequence (e.g., peptide **2**) with no effect on in vitro acitivity.[12]

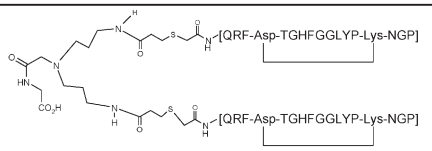
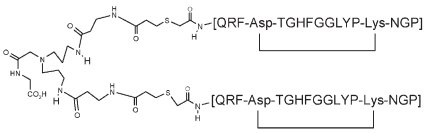
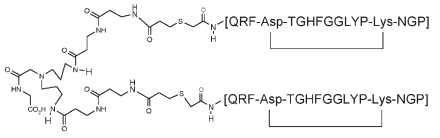
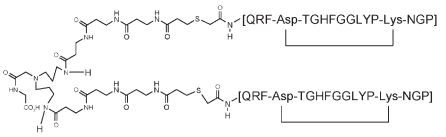
In Vivo Activity of Peptide Dimers. Since the anti-FcRn peptide family binds to human FcRn and not to mouse or rat FcRn,[11] we studied the peptide analogues using transgenic mice (TG32B) where the mouse FcRn and mouse β2m genes have been inactivated and replaced with human FcRn and human β2m genes. In addition, since mouse IgG does not bind to human FcRn, we monitored the half-life of human IgG, injected at time zero, during treatment with peptide or vehicle injections. The standard protocol for this IgG catabolism experiment involved injection of 500 mg/kg hIgG at time zero, followed by peptide injections at 24, 48, 72, and 96 h. We found previously that peptide **1** was effective at increasing the catabolism of hIgG in this model in a dose dependent manner.[11] We therefore compared different dimeric peptide analogues using this in vivo assay and correlated in vitro activity and/or structure with in vivo efficacy.

Peptide dimers were synthesized with varying synthetic methods and varying linkers. In the case of peptides **25** and **1**, the same peptide monomer was linked using different chemistry which resulted in equivalent in vitro activity (Table 2). Peptides **25** and **1** were compared in the IgG catabolism experiment, and it was found that the different linker chemistries did not affect in vivo efficacy, indicating that the in vitro assay results were predictive of the in vivo outcome (Figure 6).

The substitution of *p*-guanylphenylalanine for histidine in monomeric peptide **13** generated a peptide capable of binding FcRn with equal affinity at pH 6 and pH 7.4, and this was the most potent monomeric peptide binder of FcRn at pH 7.4 (Table 1). The dimeric form of peptide **13** was synthesized with a succinyl linker (peptide **28,** Table 5) to test the activity of a dimeric peptide optimized for binding to FcRn at pH 7.4. Peptide **28** was tested in vivo in comparison to peptide **1** which possesses the same dimeric linkage (Table 5). It was found that peptide **28** had approximately equivalent in vivo activity as peptide **1** and that the pH-7.4 optimized binding characteristics of peptide **28** did not further improve the activity of the peptide in vivo (Figure 7).

In vitro studies of the peptide monomers suggested that substitution of Thr4 with *N*-methylalanine enhanced the stability of the peptide motif to the model protease subtilisin.[12] However, this modification also led to a loss in in vitro activity of ∼3-fold with respect to the monomeric peptide (peptide **11** vs **12**, Table 1) and ∼5-fold with respect to a dimeric peptide (**22** vs **23**, Table 2). It was unclear whether this stabilizing substitution would be advantageous in vivo given the loss in in vitro activity. Substitution of Thr for NMeAla in the context of the dimer did not enhance the

Table 2. In Vitro Activity of Dimeric Peptides of Peptide Monomer **14** Linked in the N-Terminus-to-N-Terminus Orientation[a]

Peptide No.	Sequence[1]	IC_{50} IgG Competition ELISA assay nM	X-Fold Enhancement over the monomer
16		600 ± 260 (n=3)	37
17		2 400 ± 990 (n=3)	9.2
18		1 100 ± 550 (n=3)	20
19		710 ± 85 (n=3)	31
20		580 ± 340 (n=3)	38

[a] (1) All peptides are amidated at the C-terminus. All peptides are cyclic through amide bonds (Asp-Lys).

efficacy of the peptide in the IgG catabolism experiment (peptides **23** and **1**, Figure 7). Note that peptide **22**, which possessed the valerolactam substitution, is equally active to peptide **1** in vivo (data not shown). These data suggest that the potential cleavage site previously identified with subtilisin does not contribute significantly to the breakdown and/or activity of the peptide in vivo.

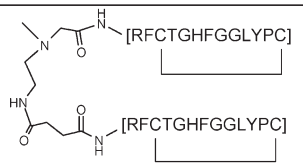
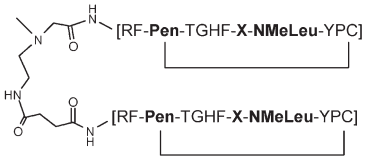
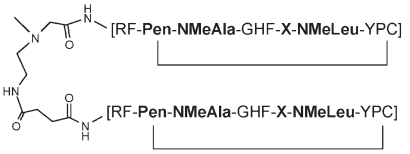
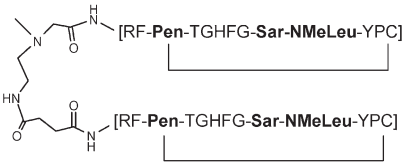
Unlike disulfide-cyclized peptides which may be reduced under certain physiological conditions, lactam-cyclized peptides are potentially more stable in vivo. We generated the most potent lactam-cyclized peptide dimer **24** and tested this molecule in vivo to determine whether or not the lactam cyclization would provide additional in vivo stability and therefore in vivo activity. Because of the relatively weak affinity of the lactam monomer **15** for FcRn, dimeric peptide **24** was approximately 20-fold weaker in vitro than peptide **1**. Peptide **24** was less active than peptide **1** in the IgG catabolism experiment, most likely a result of its weaker affinity (Figure 6).

Discussion and Conclusion

Chemical optimization of peptides derived from phage display libraries led to the discovery of peptide **1**. Key to this optimization process was dimerization of the most potent peptide monomer which increased the in vitro inhibitory activity by more than 200-fold for the most potent compounds. Dimerization was used as a tool to boost inhibitory activity to exploit well-known avidity effects of multiple binding interactions, a strategy that has been successfully applied by nature[14] and in drug development.[15,16] With respect to FcRn, since IgG is dimeric, it was hypothesized that a 2:1 interaction (2IgG−1FcRn) may be required for effective recycling of IgG in the FcRn pathway.[17,18] Therefore, a bivalent inhibitor such as peptide **1** may be better suited to inhibit the IgG recycling process in vivo.

The synthesis of disulfide- or lactam-containing peptide dimers can be challenging and a lengthy process. Others have used various strategies to generate such peptide dimers, and some examples include the coupling of unprotected peptide disulfides with bis-*N*-hydroxysuccinimide esters[19,20] or aminooxy/aldehyde chemistry[21,22] and the synthesis of a peptide dimer on-resin using lysine as a branch point, followed by sequential disulfide bond formation using orthogonal protecting groups.[23] We describe herein three different methods to generate the anti-FcRn peptide dimers, each of which has its advantages and disadvantages depending on the peptide being synthesized. Peptide dimerization using thiol/alkyl halide chemistry was an efficient method, but its use was limited in the current study because disulfide bonds were present in the most active peptides. The use of this methodology resulted in shuffling of the preformed disulfide bonds within the peptide monomers. The reductive alkylation method was the most

Table 3. In Vitro Activity of Dimeric Peptides of Various Monomeric Peptides Linked in the N-Terminus-to-N-Terminus Orientation, Using a Common Alkyl/Amide Linker[a]

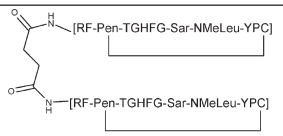
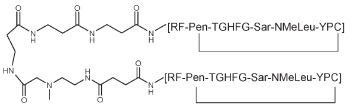
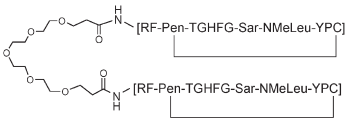
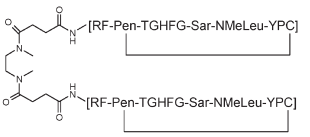
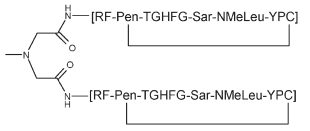
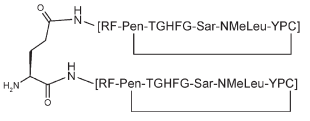
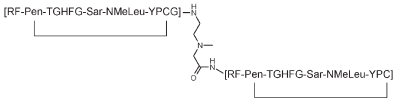
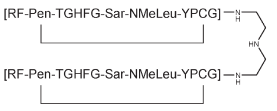
Peptide No.	Sequence[1]	Dimer of Peptide No.	IC_{50} IgG Competition ELISA assay nM	X-Fold Enhancement over the monomer
21		**10**	3 000 ± 670 (n=3)	12
22		**11**	3.9 ± 2.9 (n=3)	110
23[2]		**12**	18 ± 12 (n=3)	61
24		**15**	50 ± 31 (n=3)	38
25		**2**	2.6 ± 1.0 (n=12)	200

[a] (1) All peptides are amidated at the C-terminus. "Pen" denotes a L-penicillamine amino acid. "Sar" denotes sarcosine;. "NMeLeu" denotes a *N*-methylleucine amino acid. "NMeAla" denotes a *N*-methylalanine amino acid. "X" denotes the valerolactam dipeptide (*R*)-3-amino-2-oxo-1-piperidineacetic acid. (2) Peptide **23** was isolated at approximately 90% purity.

suitable method for initial synthesis of a peptide dimer that contained disulfide-cyclized monomeric peptides. This method was used in the synthesis of many types of dimers including various linkers and was the most reliable method to generate the dimers. This method involved the synthesis of monomeric peptides and formation of the disulfide bonds prior to dimerization with dimerization conditions that did not lead to disulfide shuffling. For difficult-to-synthesize peptides, this method was also preferred because it allows for purification of the monomeric peptides prior to dimerization. Concomitantly, the primary disadvantage of this approach is the requirement to handle two separate monomeric peptides, each with its own reactive amine or aldehyde, which adds to the labor cost of this method. Lastly, this method is also limited to peptides that do not have other free amino groups (e.g., Lys) that could be available to react with the aldehyde and complicate the purification process. Dimerization of the crude peptides on-resin was the third method used to generate peptide dimers.[13] This method was the simplest and linked adjacent amino termini on the resin using a difunctional acid linker. One of the limitations of this method was the requirement for a relatively pure peptide on resin (e.g., >70%) and for controlled oxidative conditions for intramonomer disulfide bonds. In the case of the anti-FcRn peptides, by holding the pH near 7, quantitative oxidation to the intramonomeric disulfides was achieved.

We described the synthesis of dimeric forms of anti-FcRn peptides and their structure−activity relationships using both in vitro and in vivo assays. Three different peptide dimerization methods were described where each method possesses inherent advantages depending on the particular peptide dimer to be synthesized. It was found that the synthesis of peptide dimers can increase the in vitro activity of the monomeric peptides by more than 200-fold. Structure−activity relationships of the peptide dimers demonstrated that the nature and orientation of the linker joining the two peptide

Table 4. In Vitro Activity of Dimeric Peptides of Monomeric Peptide **2** Using Various Linkers and Orientations[a]

Peptide No.	Sequence[1]	Peptide Orientation	IC_{50} IgG Competition ELISA assay nM	X-Fold Enhancement over the monomer
1		N-to-N	2.4 ± 1.4 (n=13)	220
26		N-to-N	2.4 ± 1.1 (n=3)	220
27		N-to-N	1.6 ± 0.46 (n=3)	330
29		N-to-N	4.8 ± 2.7 (n=3)	110
30		N-to-N	5.2 ± 1.3 (n=3)	100
31		N-to-N	4.7 ± 2.4 (n=3)	110
32		N-to-N	5.0 ± 3.1 (n=3)	100
33		N-to-N	4.6 ± 2.9 (n=3)	110
34		N-to-N	5.6 ± 3.7 (n=3)	90
35		N-to-C	3.9 ± 2.4 (n=3)	130
36		C-to-C	4.2 ± 0.6 (n=3)	120

[a] (1) All peptides are acetylated at the N-terminus and amidated at the C-terminus unless otherwise noted. "Pen" denotes a L-penicillamine amino acid. "Sar" denotes sarcosine. "NMeLeu" denotes a *N*-methylleucine amino acid. "NMeAla" denotes a *N*-methylalanine amino acid. "X" denotes the valerolactam dipeptide (*R*)-3-amino-2-oxo-1-piperidineacetic acid.

monomers can have a minor effect on the potency of the peptide, although no clear trend emerged from these studies. Additional characterization of the three-dimensional structures of these peptides will be required to better understand these effects. Through these studies, it was found that peptide **1** represented an optimized dimeric FcRn inhibitor that may have utility in the treatment of autoimmune diseases.

Experimental Section

General procedures for all peptide synthesis, purification, and analysis were performed as described previously.[12] All peptides were isolated with a purity of ≥95% except for peptide **23** which was isolated at approximately 90% purity, as determined by analytical reversed-phase HPLC using a C18 column (Jupiter, Phenomenex) and gradients of acetonitrile in water with 0.08% TFA and 0.02% formic acid. Peptide identity was confirmed using electrospray mass spectrometry (Mariner ES-TOF). See Supporting Information for all calculated and observed MS data and for analytical RP-HPLC chromatograms of peptide analogues used in animal studies.

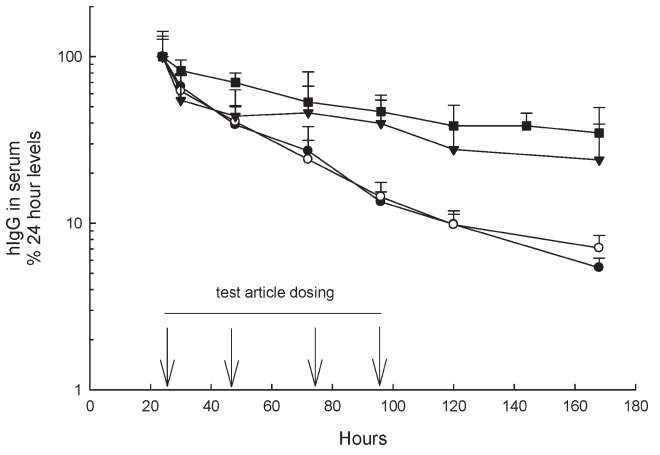
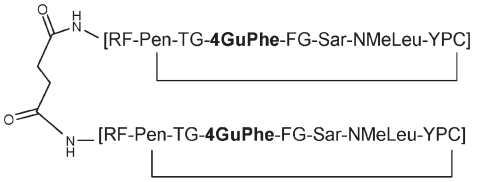
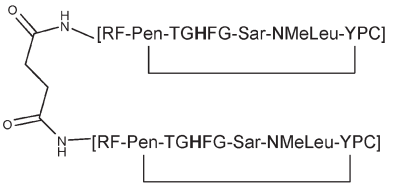


Figure 6. Effect of peptide **25** (filled circles), peptide **24** (filled triangles), peptide **1** (open cirlces), or vehicle (filled squares) on hIgG catabolism in TG32B mice. Animals were treated with hIgG at $t = 0$, followed by four daily intravenous doses of test article at $t =$ 24, 48, 72, and 96 h at a dose of 2.5 mg/kg for peptides **25** and **1** and at 5 mg/kg for peptide **24**.

Synthesis of Peptide Dimers Using Thiol/Alkyl Halide Chemistry. Bromoacetylated Peptide 4. Bromoacetylated peptide **4** was synthesized by reacting the free α-amino group of the protected lactam-cyclized peptide resin with 4 equiv of bromoacetyl bromide (Sigma-Aldrich, St. Louis, MO) and 8 equiv of DIEA (Sigma-Aldrich, St. Louis, MO) in DMF. After 1 h, the resin was washed with DMF, followed by DCM, and then cleaved from the resin and purified by HPLC as described previously.

Bis-thiol Linkers 5−8. The bis-thiol linkers **5**−**8** were synthesized by reacting NH_2-Gly-2-chlorotrityl resin (Novabiochem, San Diego, CA) with 2 equiv of *N*,*N*-bis(*N*′-Fmoc-3-aminopropyl)glycine potassium hemisulfate (Chem-Impex, Wood Dale, IL) in the presence of 2 equiv of PyBOP (Novabiochem, San Diego, CA) and DIEA in DMF for 18 h. The Fmoc protecting group was removed with two 10 min treatments of 20% piperidine in DMF. For linker compounds **6**−**8**, β-alanine residues were also incorporated as spacer units: Fmoc-β-Ala-OH (Novabiochem) was coupled to the resin using PyBOP and DIEA. After the Fmoc protecting group was removed with 20% piperidine in DMF, either another β-alanine spacer unit was coupled or the bis-thiol linker was incorporated by reacting the free N-terminal amine resin with 2 equiv of *N*-succinimidyl-*S*-acetylthioproprionate (SATP; Pierce, Rockford, IL) and 4 equiv of DIEA for 18 h. The *S*-acetyl protecting group was removed on resin using a degassed solution containing a 1:0.4 mixture of DMF and buffer A for 18 h (buffer A: 1 M hydroxylamine hydrochloride, 40 mM sodium phosphate, pH 7.5, 50 mM EDTA). Each resin was washed with DMF, DCM and cleaved from the resin with a 50% solution of TFA in DCM with

Table 5. In Vitro Activity of a Dimeric Peptide **28** in Comparison to Dimer **1**[a]

Peptide No.	Sequence[1]	Dimer of Peptide No.	IC_{50} IgG Competition ELISA assay nM	X-Fold Enhancement over the monomer
28		**13**	20 ± 9.2 (n=5)	50
1		**2**	2.4 ± 1.4 (n=13)	220

[a] Information for superscript number: (1) All peptides are amidated at the C-terminus. "Pen" denotes a L-penicillamine amino acid. "Sar" denotes sarcosine. "NMeLeu" denotes a *N*-methylleucine amino acid. "4GuPhe" denotes a 4-guanyl-phenylalanine amino acid.

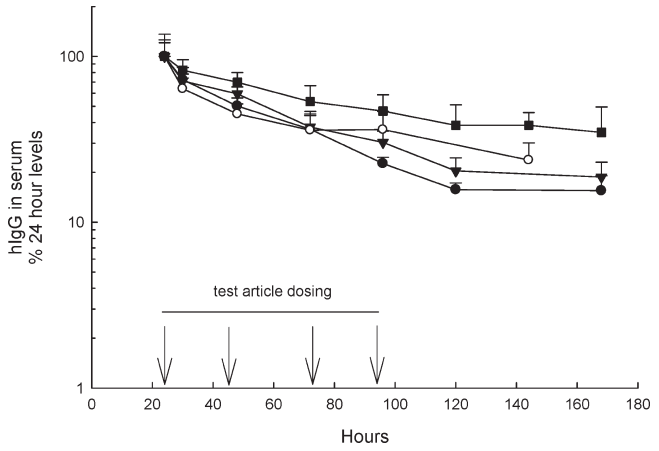


Figure 7. Effect of peptide **28** (filled triangles), **23** (open circles), **1** (filled circles), or vehicle (filled squares) on hIgG catabolism in TG32B mice. Animals were treated with hIgG at $t = 0$, followed by four daily intravenous doses of test article at 1 mg/kg at $t = 24$, 48, 72, and 96 h.

2% triisopropylsilane for 15 min. The crude linker molecules were purified as described previously for the peptides.

Thiol-Containing Lactam Peptide 9. The crude lactam-cyclized peptide resin with a free N-terminal amine was reacted with 2 equiv of SATP in DMF for 2 h. The *S*-acetyl protecting group was removed as described above, followed by cleavage from the resin and subsequent purification as described above.

Lactam-Containing Peptide Dimers 16−19. The peptide dimers were generated using bis-thiol linkers by reacting 1 equiv of the purified bis-thiol linker with a 2 equiv of peptide **1** in DMF with 10% water and 50% 100 mM sodium phosphate, pH 7.5. After 18 h, the crude reaction mixture was purified by reversed phase HPLC as described previously.

Lactam-Containing Peptide Dimer 20. Peptide **20** was synthesized by reacting 1 equiv of bromoacetylated peptide **4** with peptide **9** in 50 mM phosphate buffer, pH 7.5. After 18 h, the crude reaction mixture was purified by reversed phase HPLC as described previously.

Synthesis of Peptide Dimers 21−27 and 35−36 by Reductive Alkylation. Synthesis of Peptide Aldehydes. Peptide N-terminal aldehydes (Figure 3) were synthesized by reacting the unprotected amine of the N-terminal amino acid with 5 equiv of succinnic anhydride (Sigma-Aldrich, St. Louis, MO) in the presence of DIEA in DMF for 2 h. The resin was washed with DMF and treated with 2,2-dimethyl-1,3-dioxolane methamine (Sigma-Aldrich, St. Louis, MO) in the presence of PyBOP and DIEA for 2 h. Cleavage of the crude peptide from the resin, followed by cysteine oxidation and purification as described previously for synthesis of monomeric peptide disulfides, afforded the peptide diol. The crude peptide diol was dissolved in 33% acetic acid and treated with 2 equiv of sodium periodate (Sigma-Aldrich, St. Louis, MO) for 5 min. The reaction mixture was quenched with 20 equiv (with respect to the diol) of ethylene glycol (Sigma-Aldrich, St. Louis, MO), and after 10 min, the crude reaction mixture was diluted 3-fold with water and purified over a C18 Sep-Pak column (Waters Corp., Milford, MA), using an increasing gradient of acetonitrile in water containing 0.1% TFA, to afford the purified peptide aldehyde.

Peptide C-terminal aldehydes were synthesized using standard peptide synthesis protocols on a Fmoc-1-amino-2,3-propanediol-2′-chlorotrityl resin (Novabiochem, San Diego, CA) instead of Rink amide resin. Therefore, the resulting peptide resin contained a masked C-terminal diol. After standard peptide synthesis and cleavage from the resin, the peptide diol was oxidized first to the disulfide bonded form, then to an aldehyde as described above for N-terminal aldehydes.

Synthesis of Peptide Amines. Peptide N-terminal amines were synthesized using standard procedures. Peptide C-terminal amines were also synthesized using standard protocols except that 1,2-diaminoethane resin (Novabiochem, San Diego, CA) was used in the place of Rink amide resin. Consequently, cleavage from the resin resulted in a C-terminal ethylamine.

Synthesis of Lactam-Cyclized Peptide Amines and Aldehydes. Peptide lactam monomers used to synthesize lactam-cyclized peptide dimers such as peptide **24** were synthesized as described previously, whereby the Asp-Lys cyclization was performed on the resin, prior to cleavage from the resin.

Synthesis of Peptide Dimers by Reductive Alkylation. Peptide dimers **21−27** and **35−36** were synthesized (Figure 4) by reacting 1 equiv of peptide aldehyde with 1 equiv of amine-containing peptide at 40 mg/mL in DMF containing 2% acetic acid. After 60 min, an amount of 2 equiv of sodium cyanoborohydride (Sigma-Aldrich, St. Louis, MO) was added and the reaction was allowed proceed for 1 h. The reaction mixture was then diluted 10-fold with water and purified by HPLC.

Synthesis of Peptide Dimers 1 and 28−34 Using Diacid and Diamine Linkers. Synthesis of N-Terminally Linked Peptide Dimers. N-Terminally linked peptide dimers were synthesized as described previously,[11] by reacting the peptide resin with a bifunctional acid. This results in the covalent attachment of adjacent peptides on the resin by amide bonds via their N-termini. In the synthesis of peptides **1** and **28**, the peptide resin was treated with 0.5 equiv of succinnic acid, 1 equiv of PyBOP, and 2 equiv of DIEA. Cleavage from the resin was followed by oxidation of the disulfide bonds in 20% DMSO in 10 mM sodium phosphate, pH 7.5. Proper intramonomer disulfide bonding was confirmed using trypsin digestion/LCMS.[11]

Peptide **29** was synthesized as described above with the exception that the diacid linker used was bis-dPEG6-*N*-hydroxysuccinimide ester (Quanta Biodesigns Ltd.) and that no PyBOP was used for the coupling reaction.

Peptide **30** was synthesized as peptide **1** with the exception that the peptide−resin was treated with a large excess of succinic anhydride (Sigma-Aldrich, St. Louis, MO), which results in all peptides on the resin containing a succinate capped N-terminus. This resin was then treated with 0.5 equiv of *N*,*N*′-dimethylethylenediamine (Sigma-Aldrich, St. Louis, MO) in the presence of 1 equiv of PyBOP and 2 equiv of DIEA.

Peptide **31** was synthesized as peptide **1** with the exception that the diacid linker used was *N*-methyliminodiacetic acid (Sigma-Aldrich, St. Louis, MO).

Peptide **32** was synthesized as peptide **1** with the exception that the diacid linker used was 3,3-dimethylglutaric acid (Sigma-Aldrich, St. Louis, MO).

Peptide **33** was synthesized as peptide **1** with the exception that the diacid linker used was Boc-Asp(OH)-OH (Novabiochem, San Diego, CA).

Peptide **34** was synthesized as peptide **1** with the exception that the diacid linker used was Boc-Glu(OH)-OH (Novabiochem, San Diego, CA).

IgG Competition ELISA Assay. Peptides were assayed for their inhibition of the binding of hIgG to FcRn as described previously.[11] Briefly, neutravidin plates were coated with biotinylated soluble human FcRn (shFcRn) followed by incubation with 3 nM hIgG and various concentrations of anti-FcRn peptide at pH 6. After a 2 h incubation at 37 °C, the plate was washed and residual IgG on the plate was quantified using a peroxidase-conjugated hIgG specific Fab fragment. The concentration of peptide competitor that was capable of inhibiting 50% of the IgG−FcRn binding was designated the IC_{50}.

Surface Plasmon Resonance. SPR measurements were performed using a Biacore 3000 or T100 instrument as described previously.[11] Briefly, shFcRn was coated to the dextran surface of a CM5 sensor chip using amine coupling chemistry. The equilibrium response for each peptide dilution at either pH 6 or pH 7.4 was plotted against concentration and analyzed using the

steady state affinity model (included in the BiaEval software) to determine K_d values.

IgG Catabolism in Tg32B Mice. IgG catabolism experiments in transgenic mice were performed as described previously.[11] Briefly, TG32B mice [muFcRn (−/−), muβ2m (−/−), huFcRn (+/+), huβ2m (+/+)] were injected intravenously (iv) with 500 mg/kg hIgG (pooled subtypes) at $t = 0$ h. The mice (4 mice/group) were then injected iv at 24, 48, 72, 96 h with vehicle or peptide at doses specified in the figures and text. The vehicle was 20 mM sodium acetate, pH 5. Blood samples were taken prior to each peptide injection and at 30, 120, and 168 h except for peptide **23** for which the 120 and 168 h blood samples were switched with a 144 h blood sample. Concentrations of hIgG in serum were measured with an ELISA using goat anti-hIgG (Fab) as the capturing agent and goat anti-hIgG(Fc)-HRP for detection.

All studies in animals were conducted using protocols approved by Syntonix' Institutional Animal Care and Use Committee (IACUC), following all National Institutes of Health guidelines for the care and use of research animals.

Acknowledgment. We thank Drs. Alan Bitonti and Jennifer Dumont for helpful discussions.

Supporting Information Available: Calculated and observed LCMS data for all peptides synthesized and analytical reversed-phase HPLC chromatograms for peptides **1**, **23**−**25**, and **28**. This material is available free of charge via the Internet at http://pubs.acs.org.

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