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Design and Synthesis of Backbone Cyclic Phosphorylated Peptides: The IκB Model

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ABSTRACT:

Phosphopeptides have been used to study phosphorylation and dephosphorylation, which are key events in protein expression. Backbone cyclization has been shown to increase the stability and selectivity of peptides. Backbone cyclic peptides with conformational diversity have produced bioactive peptides with improved pharmaceutical properties, metabolic stability, and enhanced intestinal permeability. We demonstrate a successful methodology for incorporating phospho-amino acids into backbone cyclic peptides. The nuclear factor-kappa B (NF-κB) is a latent mammalian protein prototype of dimeric transcription factors that exists in all cell types and plays a pivotal role in a huge number of genes, such as those responsible for chronic and acute inflammatory diseases. To inhibit NF-κB, backbone cyclic phosphopeptides were designed and synthesized based on the conserved sequence of the Inhibitor kappa B (IκB). The peptides were screened for inhibiting IκB ubiquitylation. The best compound showed 90% inhibition at a concentration of 3 μM, and its solution structure showed similarity to a related β-catenin protein. This general methodology can be use for synthesizing cyclic phosphorylated, as well as backbone cyclic phosphorylated peptides for various biological targets. © 2008 Wiley Periodicals, Inc. Biopolymers 91: 157–168, 2009.

Keywords: *IκB; phosphorylated peptides; cyclization; NF-κB*

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INTRODUCTION

Protein–protein interactions are central to biological processes ranging from intercellular communication to programmed cell death, and they participate in many diseases, making them a large and important class of targets for human therapeutics. Small molecules are often ineffective in inhibiting protein–protein interactions, because of the large molecular area that participates in this type of interaction. Peptides in general, and cyclic peptides in particular, have been shown to inhibit protein–protein interactions and are increasingly being used for therapeutic purposes.[1–4]

Cyclization has been shown to conformationally constrain linear peptides, in addition to conferring metabolic stability, and increasing affinity and selectivity.[5] Cyclization can be performed between functional groups in the peptide, e.g., the C- and N-termini, cysteine (Cys) disulfide bonds, lysine (Lys) to aspartic acid (Asp)/glutamic acid (Glu), or side chain to terminus. However, synthetic cyclization, using side

Additional Supporting Information may be found in the online version of this article.

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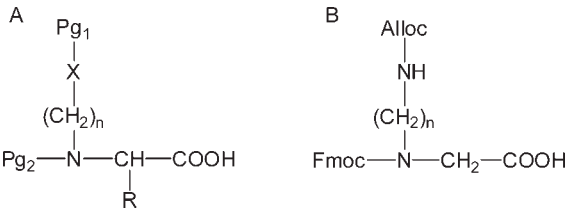

FIGURE 1 Building blocks. General structure, $Pg_{1\text{-}2}$, are orthogonal protecting groups; X, is the functional group; n, is an ω-functional alkylene chain of varying length (A); and building blocks used in this study: Fmoc-N^{α}-[ω-NH(Alloc)-ethyl] glycine-OH (n = 2); Fmoc-N^{α}-[ω-NH(Alloc)-propyl] glycine-OH (n = 3); and Fmoc-N^{α}-[ω-NH(Alloc)-hexyl] glycine-OH (n = 6) (B).

chains that are present in the native sequence may interfere with their function, especially if the residues are part of a short, conserved sequence that is necessary for binding and/or biological activity.

Backbone cyclization is done via functional groups that stem directly from the backbone leaving side chains and termini unmodified. Backbone cyclized small peptides, derived from an active site in a parent protein, can be used to study structure and function and may block protein–protein interactions with therapeutic applications.[6] Backbone cyclized peptide synthesis uses premade building blocks. These are amino acid derivatives with an alkyl chain on the backbone nitrogen that terminates with a functional group, capped with a protecting group[5] (Figure 1A). After the linear peptide is synthesized, the incorporated building blocks are deprotected and cyclized via their functional groups. The conformation of the resulting backbone cyclic peptide depends on parameters such as ring size, position, and chemistry. Conformationally diverse analogs of a chosen native sequence, which differ in these ring parameters, can be screened for biological activity. The backbone cyclization approach has been applied to various naturally occurring peptides and protein domains, such as substance P,[7] somatostatin,[8] pheromone biosynthesis activating neuropeptide,[9] and Tat,[10] resulting in selective and metabolically stable backbone cyclic peptides.

Phosphorylation and dephosphorylation are well-established elements of signal transduction, and protein expression and regulation. There are two strategies for synthesizing phosphopeptides: The building block approach and the global phosphorylation method. In the building block approach, preformed *N*-α-protected phosphorylated amino acids are incorporated into the appropriate position of the peptide sequence during synthesis and subsequently deprotected. In global phosphorylation, the serine (ser), threonine, or tyrosine residue to be phosphorylated is incorporated into the peptide sequence without side chain protection and phosphorylation is subsequently performed.[11–13]

No backbone cyclic and few cyclic phosphopeptides have been reported.[14–19] The number of cyclic phosphopeptides is small because their synthesis is challenging. The synthetic difficulties include coupling the phospho-amino acid to the resin or a previous amino acid; purification and analysis, as the peptide with the phospho-amino acid is usually negatively charged; and phospho-amino acid building blocks are significantly more expensive than regular amino acids. Nonetheless, phosphopeptides may be important for probing signal transduction events and as drug leads.

Nuclear factor-kappa B (NF-κB) is a latent transcription factor that exists in all cell types and plays a pivotal role in regulating a great number of genes. Its over-activation results in a number of chronic and acute inflammatory diseases, infection, and cancer. NF-κB activation is a paradigm for a signal transduction cascade that includes an inducible kinase and the ubiquitin-proteasome system. NF-κB forms a stoichiometric complex with Inhibitor kappa B (IκB) that binds β-transduction repeat-containing protein (β-TrCP) for NF-κB to enter the nucleus. The peptide derived from the phosphorylated conserved sequence of IκB, DS(PO_3)GXXS(PO_3), binds the β-TrCP, thereby preventing the release of NF-κB and its subsequent deleterious effects by accumulating in the nucleus and inducing relevant target genes[20–23] (Figure 2, Route A). A byproduct of NF-κB release is that IκB is also released from the complex, ubiquitylated, and sent to degradation.

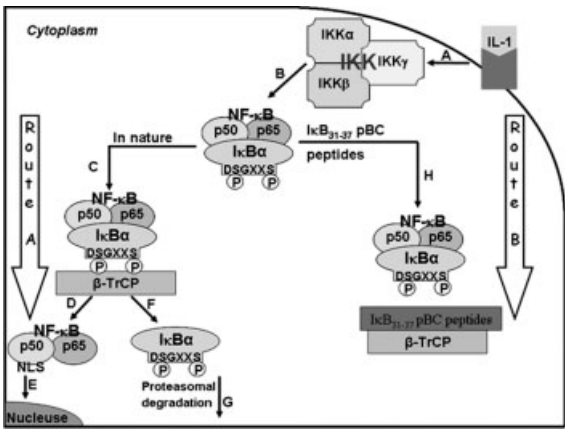

FIGURE 2 NF-κB activation pathway and inhibition by backbone cyclic phosphorylated peptides. Interleukin 1 (IL-1) activates IκB kinase (IKK) (A) that phosphorylates the conserved sequence of IκBα motif in complex with NF-κB (B). Route A: The natural pathway (C) by which the phosphorylated IκBα binds β-TrCP, causing the complex to divide into NF-κB, whose exposed nuclear-localization sequence (NLS) (D) allows it to enter the nucleus (E), and IκBα (F), which is degraded (G). Route B: In the presence of backbone cyclic phosphorylated peptides (H), the β-TrCP binding site is occupied by the peptide thus inhibiting IκB ubiquitylation.

The DpSGXXpS sequence, which includes two phosphoserine (pSer) residues, is conserved among all IκBs from *Drosophila* to human although not restricted to IκBs.[23] This sequence is also shared by other proteins such as β-catenin and the viral protein U of human immunodeficiency virus-1.[24] The structure of this sequence has been studied in β-catenin,[25] the P-IκBα$^{21-44}$ peptide,[26] and the similar DpSG*XXX*pS motif of an ATF4-phosphorylated peptide,[27] all bound to β-TrCP.

We developed a procedure for synthesizing backbone cyclic phosphorylated peptides, demonstrated here on the IκB model. Linear, precyclic and backbone cyclic phosphopeptides derived from the conserved β-TrCP binding sequence of the IκB protein were designed and synthesized using solid phase peptide synthesis (SPPS) methodology.[28] The peptides were screened for inhibiting IκB ubiquitylation, which indicated that the β-TrCP was bound to the peptide and could not participate in the process of releasing NF-κB (Figure 2, Route B). We assessed the biological activity of our peptide by measuring the degree of inhibition of the IκB ubiquitylation process. The peptides inhibited the process by which NF-κB might accumulate in the nucleus. Several backbone cyclic phosphorylated peptides inhibited IκB ubiquitylation better than the linear and precyclic analogs. The best showed 90% inhibition at a concentration of 3 μM and its solution structure was compared with the same conserved sequence in an analogous system.

RESULTS

Our general aim was to incorporate phospho-amino acids into cyclic peptides analogous to the IκB conserved sequence, DS(PO_3)GXXS(PO_3). The initial step was to synthesize linear and precyclic phosphopeptides based on the conserved sequence derived from the IκB protein, to optimize the synthetic conditions. All the peptides were screened for biochemical activity and the solution structure of the most active peptide was determined by NMR.

Design and Synthesis

We designed the peptides based on the conserved sequence of the IκB, DSGXXS. We used L-Phe and L-Ile from the *Cactus* sequence of *Drosophila* IκB protein, DSGFISG, for the nonconserved, "XX," residues in the conserved sequence.[29] All the peptides had the identical primary sequence with L-chirality.

The nomenclature of all peptides includes the mother protein (IκB), the sequence (31–37), diphosphorylation on serines (p), and whether backbone cyclization was performed including the number of methylenes of the spacer and building block alkyl chains (BC-m,n); or whether the molecule is precyclic, including the number of methylenes of the spacer and building block alkyl chains (PC-m,n).

The synthetic procedure was optimized for working with phosphorylated amino acid building blocks by solid phase peptide synthesis (SPPS) using the backbone cyclization method and 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. The linear peptide was used to identify difficulties that might occur with the specific amino acid sequence.

Phosphoserine (pSer) coupling is sluggish and was monitored by qualitative Kaiser[30] and Chloranil[31] tests and small cleavage. Coupling was achieved by both using 1-hydroxybenzotriazole (HOBt)/(2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (HBTU) and bis(trichloromethyl)carbonate (BTC).[32] In both cases, we obtained higher yields of coupling by doing two cycles of the reaction; one for 3 h and one overnight at 60°C. Coupling pSer (1.5 equiv) to natural amino acids gave best results with HOBt/HBTU coupling reagents. Coupling pSer to secondary amine building blocks (Fmoc-N$^{\alpha}$-[ω-NHallyloxycarbonyl (Alloc)-ethyl] Gly (glycine)-OH, Fmoc-N$^{\alpha}$-[ω-NH(Alloc)-propyl] glycine-OH and Fmoc-N$^{\alpha}$-[ω-NH(Alloc)-hexyl]glycine-OH (Figure 1B)[33,34]) gave best results using the BTC-activation procedure and was unsatisfactory with HOBt/HBTU.

The lactam ring was formed by an amide bond between the carboxylic acid spacer attached to the N-terminus of the peptide, and the amine functional group of the building block at the end of the N-alkyl chain after Alloc deprotection. All synthesized backbone cyclic phosphopeptides had the same primary sequence: carboxylic acid spacer-Asp-pSer-Gly-Phe-Ile-pSer-Gly-building block-NH_2 and they differed from each other only by the length of the alkyl chain of the carboxylic acid spacer ($m = 2$ and 3) and the alkyl chain of the building block ($n = 2$, 3, and 6) (Figure 3C).

The backbone cyclic phosphorylated peptides were synthesized using the tea bag method.[35] This methodology has been shown to be useful for synthesizing multiple products with minor variations. All couplings were done on bags in one polypropylene box, except for coupling building blocks and carboxylic acid spacers. Building blocks were synthesized as described.[33,34] Succinic anhydride and glutaric anhydride gave carboxylic acid spacer alkyl chains of $m = 2$ and $m = 3$, respectively (Figure 3C). Higher yields were obtained using a manual vessel,[28] so this method was used to synthesize larger amounts of active compounds required to perform biological assays and structural studies.

Mass spectrometry (MS) was determined for all peptides as expected, the results for the backbone cyclic phosphorylated peptides are presented in Table I. Figure 4 shows the mass and high pressure liquid chromatography (HPLC) spectra of biologically active IκB$_{31\text{-}37}$(pBC-2,3). The mass spectra

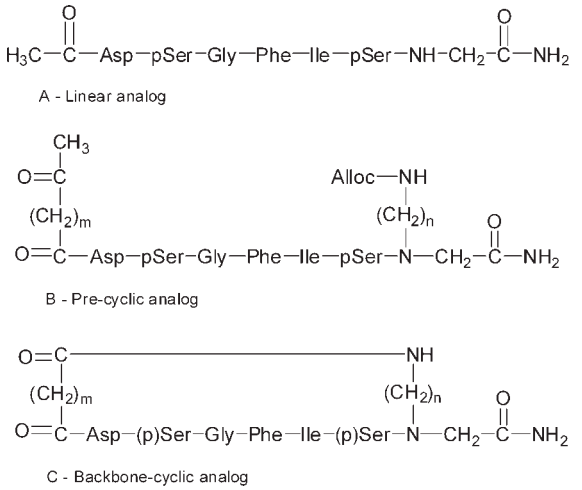

FIGURE 3 Structure of the linear peptide (A); general structure of the pre-cyclic phosphopeptides (B); and backbone cyclic peptides (C) where the serine can be phosphorylated or nonphosphorylated: (p)Ser.

and HPLC of representative backbone cyclic phosphorylated peptides are presented in the Supporting Information (Supp. Info. Figures S3–S5).

Biological Screening

The linear (Figure 3A), precyclic (Figure 3B), backbone cyclic phosphopeptides, and a nonphosphorylated backbone cyclic peptide (Figure 3C) were screened for inhibiting the specific *in-vitro* ubiquitination of pIκBα in a cell-free assay. The peptides were pre–incubated with a recombinant $SCF^{\beta\text{-TrCP}}$ complex containing stoichiometric concentrations of the recombinant proteins, F-box protein, β-TrCP, Skp1, Cul1, and Roc1, following which the $SCF^{\beta\text{-TrCP}}$ complex was used to ubiquitylate biosynthetically labeled IκBα in complex with cell-derived NF-κB.[36] Each biological assay was repeated at least twice and the assay was repeated on different synthetic batches of purified peptides. The linear, precyclic, and cyclic nonphosphate peptides were measured with an accuracy of 10%, and the cyclic phosphopeptides were measured to within 3% accuracy.

The backbone cyclic phosphopeptides $IkB_{31\text{-}37}$(pBC-2,2-3,3) inhibited pIκBα ubiquitnation by 32%–80% and 62%–90%, at concentrations of 0.3 μM and 3 μM, respectively (Figure 5 and Table II). Backbone cyclic phosphopeptide $I\kappa B_{31\text{-}37}$(pBC-2,6), with a longer six-methylene alkyl chain building block, showed less inhibition (26% inhibition at a concentration of 3 μM). The most active backbone cyclic phosphopeptide, $I\kappa B_{31\text{-}37}$(pBC-2,3), inhibited ubiquitination

Table I A Detailed Description of all the Backbone Cyclic Phosphorylated Peptides Sorted According to Ring Size

Compound	*m*	*n*	Ring Size	MS-Calc.	MS-Obs.	Purity (%)
$I\kappa B_{31\text{-}37}$(pBC-2,2)	2	2	26	965.8	964.8	98
$I\kappa B_{31\text{-}37}$(pBC-3,2)	3	2	27	979.8	978.9	99
$I\kappa B_{31\text{-}37}$(pBC-2,3)	2	3	27	979.8	978.8	100
$I\kappa B_{31\text{-}37}$(pBC-3,3)	3	3	28	993.8	992.9	100
$I\kappa B_{31\text{-}37}$(pBC-2,6)	2	6	30	1021.9	1020.7	100

m and *n* refer to the number of methylenes in the alkyl spacer and building block, respectively (Figure 3C). MS was done using MALDI technique in negative mode. Purity was determined by analytical HPLC. The basic sequence of $IkB_{31\text{-}37}$ is DSGFISG (see Figure 3).

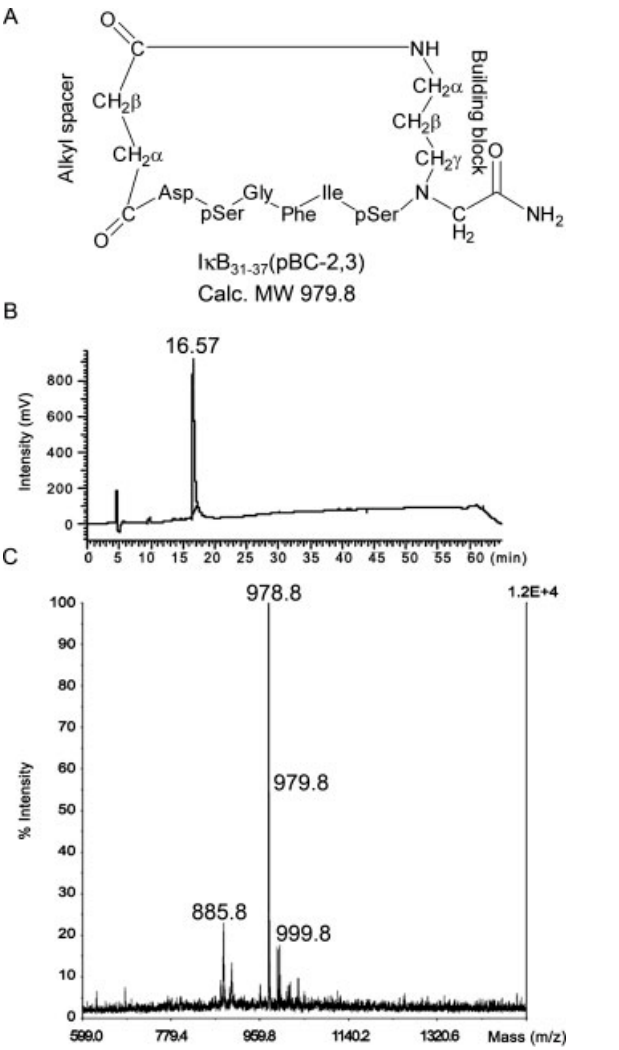

FIGURE 4 Characterization of $I\kappa B_{31\text{-}37}$(pBC-2,3) peptide. (A) Structure of $I\kappa B_{31\text{-}37}$(pBC-2,3). (B) Analytical HPLC of $I\kappa B_{31\text{-}37}$ (pBC-2,3) (run with system B). (C) MS – The peaks are: 978.8 m/z – molecular weight (MW) of $I\kappa B_{31\text{-}37}$(pBC-2,3); 999.8 m/z – MW of $I\kappa B_{31\text{-}37}$(pBC-2,3) + Na; and 885.8 m/z – a fragmentation of $I\kappa B_{31\text{-}37}$ (pBC-2,3) with cleavage of H_3PO_4 (MALDI, negative mode).

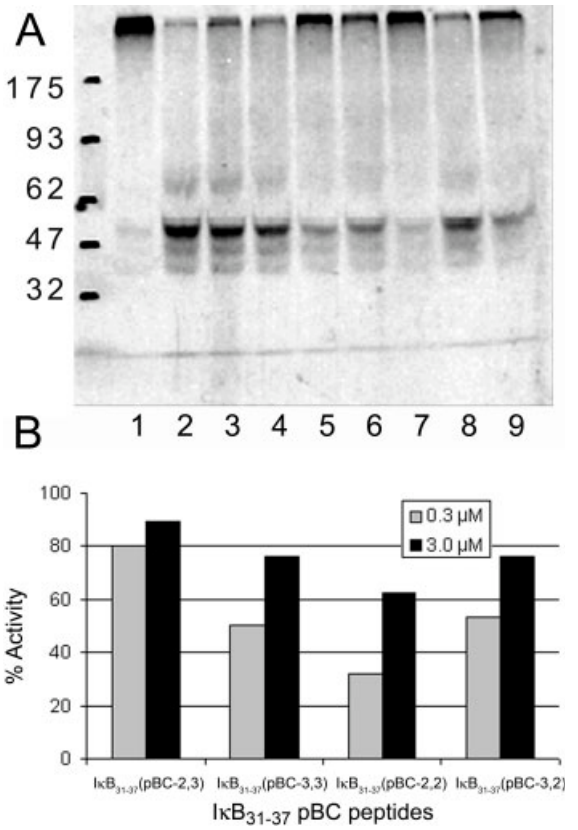

FIGURE 5 Screening IκB$_{31\text{-}37}$(pBC-2,2-3,3) peptides for inhibition of IκBα ubiquitylation by recombinant SCF$^{\beta\text{-TrCP}}$ complex. The IκB$_{31\text{-}37}$(pBC-2,2-3,3) peptides were screened for their ability to inhibit IκBα ubiquitylatin at: 0.3 μM and 3 μM. (A) In-vitro ubiquitylation of NF-κB-associated ^{35}S IkBα in the presence of various synthetic peptides (see Materials and Methods). Lane 1, no peptide; Lane 2, IκB$_{31\text{-}37}$(pBC-2,3) 3 μM; Lane 3, IκB$_{31\text{-}37}$(pBC-2,3) 0.3 μM; Lane 4, IκB$_{31\text{-}37}$(pBC-3,3) 3 μM; Lane 5, IκB$_{31\text{-}37}$(pBC-3,3) 0.3 μM; Lane 6, IκB$_{31\text{-}37}$(pBC-2,2) 3 μM; Lane 7, IκB$_{31\text{-}37}$(pBC-2,2) 0.3 μM; Lane 8, IκB$_{31\text{-}37}$(pBC-3,2) 3 μM; Lane 9, IκB$_{31\text{-}37}$(pBC-3,2) 0.3 μM. (B) Percentage of inhibition was determined by the biological assay system (Materials and Methods).

by 80% and 90% at concentrations of 0.3 μM and 3 μM, respectively, with an IC$_{50}$ of 0.03 μM (Table II).

Inhibition was determined for the linear analog, IκB$_{31\text{-}37}$ (Linear) (Figure 3A), and two precyclic analogs [IκB$_{31\text{-}37}$ (pPC-2,3) and IκB$_{31\text{-}37}$(pPC-3,3), Figure 3B], all having the same sequence. The precyclic analogs had a spacer and building block, but the final cyclization step was not performed. The linear analog and the precyclic analogs were less active than the cyclic IκB$_{31\text{-}37}$(pBC-2,3) [14% inhibition at a concentration of 3 μM for the linear analog and 24% and 33% inhibition at a concentration of 3 μM for the precyclic analogs IκB$_{31\text{-}37}$(pPC-2,3) and IκB$_{31\text{-}37}$(pPC-3,3), respectively]. The nonphosphorylated analog, IκB$_{31\text{-}37}$(BC-2,3), was inactive showing that phosphorylation was required for activity. A reference minimal size peptide (DpSGLDpSM) which spans the signaling phosphorylation site, inhibited ubiquitination with an IC$_{50}$ of 15 μM.[21]

Nuclear Magnetic Resonance

NMR spectroscopy was used to obtain the molecular structure of IκB$_{31\text{-}37}$(pBC-2,3). The correlation spectroscopy (COSY), total correlation spectrometry (TOCSY), and nuclear Overhauser effect spectroscopy (NOESY) spectra of IκB$_{31\text{-}37}$(pBC-2,3) were acquired under the same experimental conditions (Supp. Info. Figure S1). All the peaks and hydrogens were accounted for. The peptide, spacer, and building block moieties were well-resolved in the two-dimensional spectra and assigned (Table III). The NOESY spectrum had 69 peaks of which 34 were intraresidual, 10 were sequential, 5 were $i,i+2$, 2 were $i,i+3$, and 18 were between the spacer-building unit and the peptide moiety (Supp. Info. Table S1). The backbone showed HN—HN interactions between Gly 3 and phenylalanine (Phe) 4, isoleucine (Ile) 5 and Ser 6, and the HN-to-side chain interactions of these residues were also seen.

The three-dimensional structure of IκB$_{31\text{-}37}$(pBC-2,3) was derived using a starting ensemble of 50 structures. Of these, 42 (84%) had no NOE violations, bond lengths that were correct to within 0.05 Å, and angles and dihedral violations within 5° of ideal values. The calculated ensemble of conformers (see Figure 6) had a backbone RMSD of 0.7 STD 0.3 Å and a heavy atom RMSD of 1.7 STD 0.4 Å.

The secondary elements showed a secondary bend around Gly 3 – Ile 5 in over half of the minimized low energy conformations (Supp. Info. Figure S2).

The full columbic electric potential distribution was calculated and the surfaces of the +3 kT/e (blue) and −3 kT/e

Table II Inhibition of Binding Between β-TrCP and IκB (μM)

Compound[a]	% Inhibition at 3 μM	% Inhibition at 0.3 μM
IκB$_{31\text{-}37}$(Linear)[b]	14	20
IκB$_{31\text{-}37}$(pPC-2,3)[b]	33	16
IκB$_{31\text{-}37}$(pPC-3,3)[b]	24	17
IκB$_{31\text{-}37}$(BC-2,3)[b]	0	0
IκB$_{31\text{-}37}$(pBC-2,2)[c]	62	32
IκB$_{31\text{-}37}$(pBC-3,2)[c]	76	53
IκB$_{31\text{-}37}$(pBC-2,3)[c]	90	80
IκB$_{31\text{-}37}$(pBC-3,3)[c]	76	55

[a] See Figure 3 for nomenclature.

[b] Error estimates are 10% for the active backbone cyclic phosphorylated peptides.

[c] Error estimates are 3% for the active backbone cyclic phosphorylated peptides.

Table 3 ^{1}H NMR Chemical Shift Assignment (ppm) of IκB$_{31\text{-}37}$(pBC-2,3) in 10% D_2O in Water

	HN	Hα	Hβ	Others
Dicarboxylic acid spacer[a]		2.75, 2.63	2.39	
Building block[a]	8.09	3.12, 3.00	1.71	Hγ CH_2 3.48, 3.37
Asp 1	8.50	4.53	2.75, 2.64	
Ser 2	8.63	4.26	3.93	
Gly 3	8.39	3.76, 3.54		
Phe 4	7.87	4.45	3.04, 2.85	Hδ 7.07; Hε 7.24; Hζ 7.16
Ile 5	7.99	4.01	1.65	Hγ CH 1.27; CH_3 1.00; Hδ CH_3 0.70
Ser 6	8.68	4.84	3.90	
Gly 7		3.86		

[a] See Figure 4A for nomenclature.

(red) potentials (see Figure 7) show an obvious and expected affinity for positive charge with a negative isosurface extended along the length of the peptide with bulbs around the phospho moieties. The amidated Gly 7 and the Phe 4 side chain are in the middle surrounded by a positive isosurface.

DISCUSSION

Phosphorylation and dephosphorylation are well-established elements of signal transduction, protein expression, and regulation, and they participate in cell cycle control. Phosphopeptides may be useful for studying these processes. Here we incorporate phospho-amino acids into backbone cyclic peptides based on the primary sequence of a conserved motif of IκB, DSGFISG, as a model.

From a synthetic point of view, the most difficult steps in the synthesis were coupling pSer and coupling sterically hindered building blocks to the resin. This was overcome by using two cycles of HOBt/HBTU for coupling to natural amino acids and by using two cycles of BTC activation for coupling to secondary amines, both at elevated temperatures. The tea bag method[35] is useful for efficiently synthesizing diverse peptides. Nevertheless, higher yields were obtained using a manual vessel so this method was used to produce large amounts of product. The yields obtained for peptides synthesized using manual vessels were around 20% with over 98% purity.

Several of the backbone cyclic phosphopeptides inhibited the specific *in-vitro* ubiquitination of pIκBα. We found that peptides that had smaller ring sizes [26–28 atoms – IκB$_{31\text{-}37}$

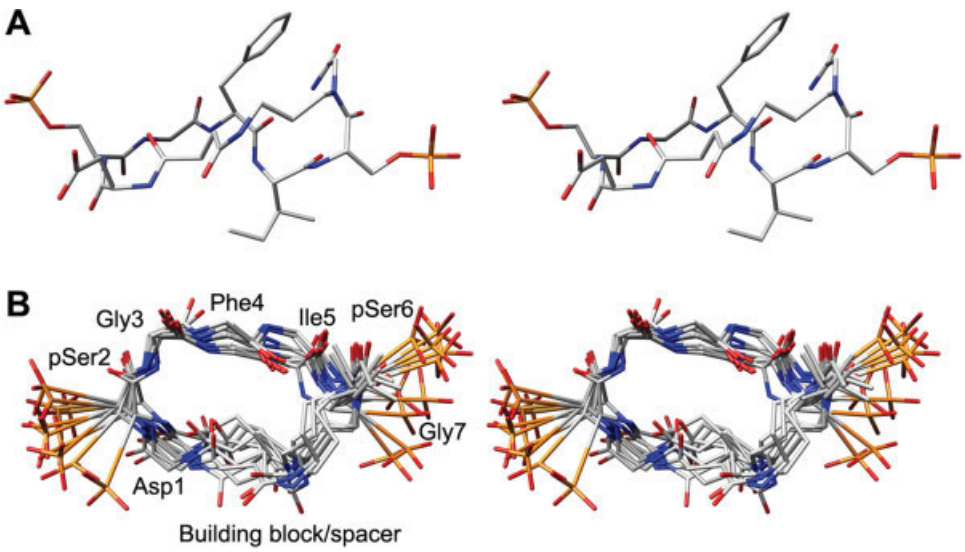

FIGURE 6 Stereo view of NMR-derived low-energy representative conformation of IκB$_{31\text{-}37}$(pBC-2,3) peptide (A) and another view of backbone and phosphates of 10 low energy structures, RMSD 0.7 STD 0.3 Å (backbone), and 1.7 STD 0.4 Å (heavy atom) (B). Figure prepared with Chimera.[54]

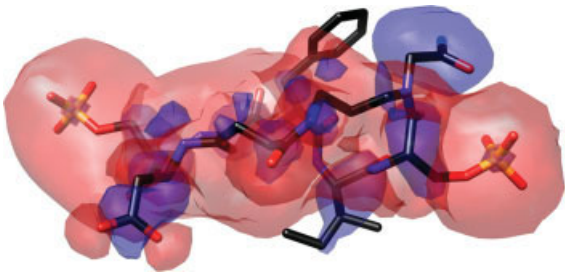

FIGURE 7 Electrostatic potential isosurfaces of 3 kT/*e* (blue) and −3 kT/*e* (red) of IκB$_{31\text{-}37}$(pBC-2,3) peptide. Figure prepared with Chimera.[54]

(pBC-2,2-3,3)] inhibited the ubiquitylation of IκB better than peptides with larger ring sizes [30–31 atoms – IκB$_{31\text{-}37}$ (pBC-2,6) and data not shown] suggesting a possible significance of restricted ring size for activity (Figure 3C, Table II, and Results section). The smaller ring reduces the number of conformations that the peptide can adopt, presumably constraining the peptide to an active conformation.[37] IκB$_{31\text{-}37}$ (pBC-2,3) (Figure 3C, ring size $m = 2$, $n = 3$) showed the highest inhibition of 90% at a concentration of 3 μM.

The linear analog was less active than the most active backbone cyclic phosphopeptide. This is probably due to the flexibility of the linear peptide as opposed to the restrained cyclic peptide. The precyclic analogs, [IκB$_{31\text{-}37}$(pPC-2,3) and IκB$_{31\text{-}37}$(pPC-3,3)], were less active than the most active backbone cyclic phosphopeptide, but more active than the linear peptide. This can be attributed to a reduction in flexibility due to the effective N-alkylation.[38] The nonphosphorylated backbone cyclic peptide, IκB$_{31\text{-}37}$(BC-2,3), was completely inactive, as expected. These results are in agreement with the results of Yaron et al., that showed that peptides required phosphorylation at both serines to inhibit ubiquitination effectively.[21]

The NMR-derived structure of IκB$_{31\text{-}37}$(pBC-2,3) showed a defined backbone, seen in the low RMSD value, as expected for a cyclic restrained molecule.[39] The Phe 4 and Ile 5 side chains adopted axial-like positions on the ring presumably due to ring strain induced by the charge–charge repulsion of the two phospho-groups. The building block and spacer moieties show less rigidity, as common in backbone cyclic peptides, as they are not stabilized by side chains.

As there is no crystal structure available for the N-terminal sequence of IκB, we superimposed the low energy structure of IκB$_{31\text{-}37}$(pBC-2,3) on the backbone of the conserved sequence of β-catenin in complex with β-TrCP (PDB accession code 1P22)[25] (Figure 8A). β-catenin has the same binding motif as IκB, in this case DSGIHS, which is responsible for recognition and binding by β-TrCP. IκB$_{31\text{-}37}$(pBC-2,3) fits into the slightly elongated positive binding pocket of β-catenin in the β-TrCP (Figure 8B). The side chain of Phe 4 fits into a hydrophobic pocket of β-TrCP, which is occupied by Ile in the complex with β-catenin (Figure 8B). Although the experimental conditions differ from those of the crystallization[25] and NMR[26,27] conditions in which this motif has been studied, the bend in the conserved sequence is apparent in all structures. The distance between the two phosphate groups in the low energy structure (see Figure 6) was 17.8 Å, corresponding to the same distance of 17.8 Å found in a peptide derived from the ATF4 motif complexed with β-TrCP and a distance of 17.1 Å in the consensus motif in the β-catenin peptide.[27] The negative potential of IκB$_{31\text{-}37}$(pBC-2,3) may serve for long-range affinity towards the positive binding pocket of the β-TrCP and the hydrophobic moieties potentially form hydrophobic interactions with the β-TrCP leucine (Leu) residues 311, 351, and 472, and alanine (Ala) 434 that are known to be part of the binding pocket of β-TrCP.[25–27] This complementarity suggests that the activity of IκB$_{31\text{-}37}$(pBC-2,3) may be similar to that of β-catenin.

We present the syntheses of novel backbone cyclic phosphopeptides, together with a general methodology for

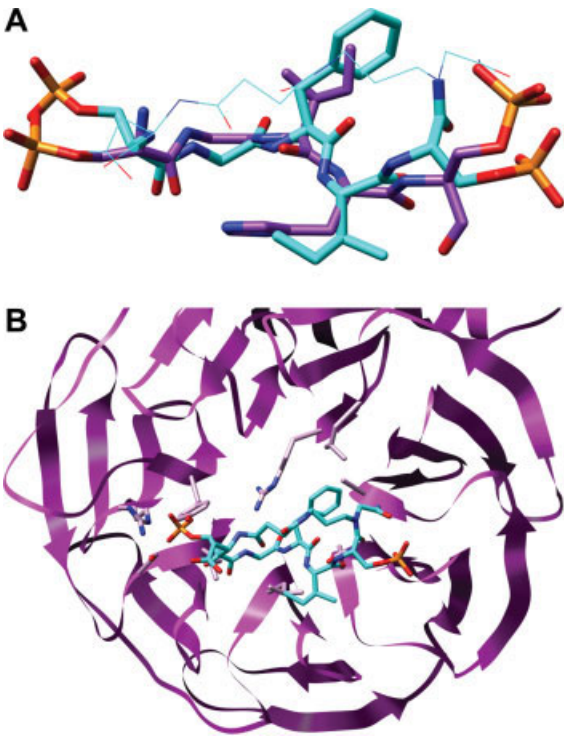

FIGURE 8 The conserved sequence of IκB$_{31\text{-}37}$(pBC-2,3) peptide (stick, with the building block and spacer represented by wire) superimposed on of the conserved sequence of β-catenin in the crystallographic complex structure with β-TrCP (PDB 1P22)[25] (A); the peptide in the β-TrCP binding pocket (B). Figure prepared with Chimera.[54]

coupling phospho-amino acids to regular amino acids and secondary amines. The general methodology may serve for synthesizing common natural or modified phosphorylated peptides, which may be used to great advantage to study many physiological processes of phosphorylation and dephosphorylation. Furthermore, the NMR-derived structure of the most active inhibitor was found to resemble the analogous conserved sequence of the β-catenin protein. The $I\kappa B_{31\text{-}37}$(pBC-2,3) backbone cyclic phosphopeptide was active in the low micromolar range and is a potential inhibitor of NF-κB activity.

MATERIALS AND METHODS

Hazards

Bis(trichloromethyl)carbonate (BTC) is very toxic by inhalation and causes burns. When this material is used it should be handled in the hood with extreme caution.

Reagents

All starting materials were purchased from commercial suppliers and used without further purification. Rink amide methylbenzhydrylamine (MBHA) resin was purchased from Novabiochem (catalog #A28529; lot #01-64-0037). Solid phase reactions were run at room temperature (rt) except where mentioned and were performed by manual solid phase peptide synthesis (SPPS) vessels or in tea bags by the simultaneous multiple synthesis (SMS) methodology.[28,35] The SPPS vessels were equipped with sintered glass bottoms. When tea bags SMS was implemented, the resin was sealed in 5.5 $\times$ 6.0 cm^2 polypropylene bags which were placed in polypropylene boxes. The SPPS vessel and SMS tea bags were shaken with a MilliGen 504 Big Bill shaker. All amino acids were Fmoc-protected on their N^{α} and had *tert* butyl (*t*Bu) protecting groups on their side chain functional groups, except for Fmoc-Asp(OBzl)-OH and Fmoc-Ser(PO(OBzl)OH)-OH.

General Methods

Mass Spectrometry. Mass spectra measurements were recorded on: (1) ThermoQuest Finnigan LCQ-Duo spectrometer, (2) Q-TOF II – Micromass apparatus (Micromass, UK) both using the electrospray ionization (ESI) technique and (3) Voyager-DE™ PRO Biospectrometry work station matrix assisted laser desorption ionization (MALDI) technology. Both positive and negative modes were used.

Thin Layer Chromatography. Thin layer chromatography plates were Merck silica gel $60F_{254}$ on aluminum. Visualization was achieved either with UV light at 254 nm or by spraying a solution of 1% ninhydrin in methanol and heating in an oven at 100°C.

Analytical High Pressure Liquid Chromatography. Analytical high pressure liquid chromatography was performed on a Waters 2695 system (equipped with a 2996 diode array detector) at 220 nm as well as on a Merck-Hitachi LaChrom D7000 system (equipped with L-7400 UV-Vis detector) at 220 nm, with an L-7100 pump and L-7200 autosampler. In all cases samples were analyzed on a Waters Atlantis™ dC,[18] 3 μm, 4.6 $\times$ 150 mm^2 column at 30°C or an XTerra™ RP8, 3.5 μm, 4.6 $\times$ 150 mm^2 column at 30°C or on a VYDAC 208TP™ Series C8, 5 μm, 4.6 $\times$ 250 mm^2 column at 30°C. The elution program on both systems used eluents A [0.1% trifluoroacetic acid (TFA) in triple distilled water (TDW)] and B [0.1% TFA in acetonitrile (ACN)], with a nonlinear option (system A) starting with 5% B at 30 min, 30% B at 35 min, 95% B at 40 min, and 5% B at 50 min. A linear gradient (system B) was also used, starting with 5% B and 95% A with a slope of 2%/min and a flow of 1.0 ml/min. Retention times refer to the designated system.

Purification. Preparative high pressure liquid chromatography was performed on a Merck-Hitachi 665A system (equipped with a Jasco Uvidec-100-v UV-Vis detector set at 220 nm, a preparative L-6200A intelligent pump, D-2500 chromato integrator). All the compounds were purified on a Waters Atlantis™ dC,[18] 10 μm, 10 $\times$ 150 mm^2 column at 30°C or on a VYDAC 208TP™ Series C8, 10 μm, 22 $\times$ 250 mm^2 column at 30°C. Eluting compositions were A (0.1% TFA in TDW) and B (0.1% TFA in ACN), with a nonlinear option (system A) starting with 5% B at 30 min, 30% B at 35 min, 95% B at 40 min, and 5% B at 50 min. A linear gradient (system B) was also used, starting with 5% B and 95% A with a slope of 2%/min and a flow of 9.0 ml/min. Retention times refer to the designated system.

Kaiser (Ninhydrin) Test. A sample containing 1–3 mg of resin was transferred to a glass test tube. Three drops of a solution of 2.5 g ninhydrin in 50 ml ethanol, three drops of a solution of 40 g phenol in 10 ml ethanol, and 3 drops of a solution of 1 ml 0.001*M* aqueous KCN in 49 ml pyridine were added. The solution was mixed well and placed on a block preheated to 110°C for 5 min. A positive reaction, giving the resin and the solution a purple–blue color, indicated incomplete coupling of an amino acid or deprotection of protecting groups.[30]

Chloranil Test. A sample containing 1–3 mg of resin was transferred to a test tube. Three drops of 2% acetaldehyde in dimethylformamide (DMF) and 3 drops of 2% chloranil in DMF were added. The solution was mixed well at rt for 5 min and the beads inspected. A positive reaction, giving the beads a blue color, indicated incomplete coupling of an amino acid or deprotection of protecting groups.[31]

Small Cleavage. A small amount of peptidyl resin was treated with a precooled mixture of 95% TFA, 2.5% TDW, and 2.5% triisopropylsilane (TIS). The mixture was shaken for 0.5 h at rt. The resin was removed by filtration and the solvents were evaporated by a stream of nitrogen. The residue was dissolved in ACN-TDW 1:1. The filtrated solution was analyzed by HPLC and/or MS.

Synthesis

Solid-Phase Synthesis of the Linear Phosphopeptide. A Rink amide MBHA resin (loading 0.6 mmol/g) was preswollen for 4 h in 1-methyl-2-pyrrolidinone (NMP). The Fmoc protecting groups were removed from the resin with 20% piperidine in NMP (2 $\times$

30 min). The resin was washed with NMP (5 × 2 min) and the reaction was monitored by qualitative Kaiser and chloranil tests. The Fmoc protected amino acid couplings were carried out with HOBt/HBTU -activation as follows: Fmoc protected amino acids (1.5 equiv) were dissolved in NMP, 7.5 ml, and *N,N*-diisopropylethylamine (DIEA) (1.5 equiv) and HOBt (1.5 equiv) were added. The mixture was cooled in an ice bath and HBTU (1.5 equiv) and dichloromethane (DCM) [1,2-dibromoethane (DBE) for the second Fmoc-Ser(PO(OBzl)OH)-OH coupling which was done at 60°C] 2.5 ml, was added. The mixture was preactivated by mixing for 10 min, added to the resin, and shaken for 3 h. The reagents and solvents were aspirated and replenished and shaken overnight. The resin was washed with NMP (5 × 2 min). The reaction was monitored by qualitative Kaiser and chloranil tests, and small cleavage. After the first amino acid was coupled, capping was performed with a 20 ml mixture of acetic anhydride (0.5*M*), DIEA (0.125*M*), and HOBt (0.015*M*) in DMF for 1 h, twice. The resin was washed with NMP (5 × 2 min) and DCM (2 × 2 min). After coupling each Fmoc protected amino acid, the Fmoc group was removed as described earlier, the resin was washed, and the Fmoc removal was monitored by qualitative Kaiser and chloranil tests. After the last amino acid was coupled, the Fmoc group was removed, the resin was washed and the reaction was monitored by qualitative Kaiser and chloranil tests, followed by capping, and the final cleavage was done. The polymer-bound molecules were cleaved by adding a precooled mixture of 95% TFA, 2.5% TDW, and 2.5% TIS. The mixture was kept in an ice bath for an additional 0.5 h and then shaken for 3 h at rt. The resin was removed by filtration and washed three times with small portions of TFA. The combined TFA fractions were evaporated to dryness with a stream of nitrogen, and the oily residue was precipitated in a cold solution of 30% acetic acid and 70% TDW. The precipitate was dissolved in ACN-TDW 1:1 and lyophilized. The product was purified by preparative HPLC and checked by MS and analytical HPLC its degree of purity was determined and it was sent for biological screening.

Solid-Phase Synthesis of the Precyclic Phosphopeptide. A Rink amide MBHA resin (loading 0.6 mmol/g) was preswollen for 4 h in NMP. The Fmoc group was removed. After washing, the reaction was monitored by qualitative Kaiser and chloranil tests. The building block coupling cycle was carried out with BTC-activation at 60°C as follows: Fmoc-protected amino acid (5 equiv) was dissolved in DBE and BTC (1.65 equiv) was added. The mixture was cooled in an ice bath and 2,4,6-collidine (14 equiv) was slowly added. The mixture was preactivated by mixing for 1 min, added to the resin, and shaken at 60°C for 3 h. The reagents and solvents were aspirated and replenished, and shaken overnight. The resin was washed with DCM (5 × 2 min) and NMP (2 × 2 min). After washing, the reaction was monitored by qualitative Kaiser and chloranil tests, and small cleavage. Capping was performed with a 20 ml mixture of acetic anhydride (0.5*M*), DIEA (0.125*M*) and HOBt (0.015*M*) in DMF for 1 h, twice. The resin was washed with NMP (5 × 2 min) and DCM (2 × 2 min). Following capping the Fmoc group was removed. After washing, the Fmoc removal was monitored by qualitative Kaiser and chloranil tests. The Fmoc-Ser (PO(OBzl)OH)-OH coupling cycle was carried out with BTC-activation at 60°C as described earlier. After washing, the reaction was monitored by qualitative Kaiser and chloranil tests and small cleavage. The Fmoc group was removed and after washing, the removal was monitored by qualitative Kaiser and chloranil tests. The rest of the Fmoc protected amino acid couplings were carried out with HOBt/HBTU-activation as follows: Fmoc protected amino acid (1.5 equiv) was dissolved in NMP, 7.5 ml, and DIEA (1.5 equiv) and HOBt (1.5 equiv) were added. The mixture was cooled in an ice bath and HBTU (1.5 equiv) and DCM [DBE for the second Fmoc-Ser(PO(OBzl)OH)-OH for which the coupling was done at 60°C] 2.5 ml were added. The mixture was preactivated by mixing for 10 min, added to the resin, and shaken for 3 h. The reagents and solvents were aspirated and replenished and shaken overnight. The resin was washed with NMP (5 × 2 min). The reaction was monitored by qualitative Kaiser and chloranil tests, and small cleavage. After the last amino acid was coupled. The Fmoc group was removed and, after washing, the removal was monitored by qualitative Kaiser and chloranil tests, and small cleavage. After the last amino acid was coupled, the Fmoc group was removed, the resin was washed, and the reaction was monitored by qualitative Kaiser and chloranil tests, then capping and the final cleavage were done. The polymer-bound molecules were cleaved by adding a precooled mixture of 95% TFA, 2.5% TDW, and 2.5% TIS. The mixture was kept in an ice bath for an additional 0.5 h and then shaken for 3 h at rt. The resin was removed by filtration and washed three times with small portions of TFA. The combined TFA fractions were evaporated to dryness with a stream of nitrogen, and the oily residue was precipitated in a cold solution of 30% acetic acid and 70% TDW. The precipitate was dissolved in ACN-TDW 1:1 and lyophilized. The product was purified by preparative HPLC checked by MS and analytical HPLC, its degree of purity was determined, and it was sent for biological screening.

Solid-Phase Synthesis of the Backbone Cyclic Phosphopeptides. A Rink amide MBHA resin (loading 0.6 mmol/g) was preswollen for 4 h in NMP. The Fmoc group was removed. After washing, the reaction was monitored by qualitative Kaiser and chloranil tests. The building block coupling cycle was carried out with BTC-activation at 60°C as follows: Fmoc-protected amino acid (5 equiv) was dissolved in DBE, and BTC (1.65 equiv) was added. The mixture was cooled in an ice bath and 2,4,6-collidine (14 equiv) was slowly added. The mixture was preactivated by mixing for 1 min, added to the resin, and shaken at 60°C for 3 h. The reagents and solvents were aspirated and replenished and shaken overnight. The resin was washed with DCM (5 × 2 min) and NMP (2 × 2 min). After washing, the reaction was monitored by qualitative Kaiser and chloranil tests, and small cleavage. Capping was performed with a 20 ml mixture of acetic anhydride (0.5*M*), DIEA (0.125*M*) and HOBt (0.015*M*) in DMF for 1 h, twice. The resin was washed with NMP (5 × 2 min) and DCM (2 × 2 min). Following capping the Fmoc group was removed. After washing, the Fmoc removal was monitored by qualitative Kaiser and chloranil tests. The Fmoc-Ser (PO(OBzl)OH)-OH coupling cycle was carried out with BTC-activation at 60°C as follows: Fmoc-protected amino acid (3 equiv) was dissolved in DBE, and BTC (0.98 equiv) was added. The mixture was cooled in an ice bath and 2,4,6-collidine (8.4 equiv) was slowly added. The mixture was preactivated by mixing for 1 min, added to the resin, and shaken for 3 h. The reagents and solvents were aspirated and replenished and shaken overnight. The resin was washed with DCM (5 × 2 min) and NMP (2 × 2 min). After washing, the reaction was monitored by qualitative Kaiser and chloranil tests,

and small cleavage. After washing, the reaction was monitored by qualitative Kaiser and chloranil tests, and small cleavage. The Fmoc group was removed. After washing, the Fmoc removal was monitored by qualitative Kaiser and chloranil tests. The rest of the Fmoc protected amino acid couplings were carried out with HOBt/HBTU-activation as follows: Fmoc protected amino acid (1.5 equiv) was dissolved in NMP, 7.5 mL, DIEA (1.5 equiv), and HOBt (1.5 equiv) were added. The mixture was cooled in an ice bath and HBTU (1.5 equiv) and DCM [DBE for the second Fmoc-Ser (PO(OBzl)OH)-OH for which the coupling was done at 60°C] 2.5 mL, was added. The mixture was preactivated by mixing for 10 min, added to the resin, and shaken for 3 h. The reagents and solvents were aspirated and replenished and shaken overnight. The resin was washed with NMP (5 × 2 min). The reaction was monitored by qualitative Kaiser and chloranil tests, and small cleavage. After the last amino acid was coupled the Fmoc group was removed and, after washing, the Fmoc removal was monitored by qualitative Kaiser and chloranil tests, and small cleavage. Anhydride coupling was carried out as follows: anhydride (10 equiv) was dissolved in NMP, and DIEA (10 equiv) and 4-dimethylaminopyridine (DMAP) (1 equiv) were added. The mixture was added to the resin and shaken for 3 h. The resin was washed with NMP (5 × 2 min) and DCM (2 × 2 min). The reaction was monitored by qualitative Kaiser and chloranil tests, and small cleavage. The Alloc protecting group was removed from the resin after drying the resin with a stream of argon (Ar); and the resin was washed with a solution of 1.5% acetic acid and 2.5% 4-methylmorpholine in NMP. Tetrakis (triphenylphosphine)palladium(0) (0.5 equiv) was added to this solution and the vessel was shaken in the dark for 3 h. The resin was washed with 0.5% DIEA in DMF (5 × 2 min), 0.5% sodium diethyldithiocarbamate trihydrate in DMF (5 × 2 min) and DCM (5 × 2 min). The Alloc removal was monitored by qualitative Kaiser and chloranil tests. The cyclization was performed using benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) (5 equiv) and DIEA (10 equiv) that were dissolved in DCM, added to the resin and shaken for 1 h. The resin was washed with DCM (5 × 2 min) and the final cleavage was done. The polymer-bound molecules were cleaved by adding a precooled mixture of 95% TFA, 2.5% TDW, and 2.5% TIS. The mixture was kept cool for an additional 0.5 h in an ice bath and then shaken for 3 h at rt. The resin was removed by filtration and washed three times with small portions of TFA. The combined TFA fractions were evaporated to dryness with a stream of nitrogen, and the oily residue was precipitated in a cold solution of 30% acetic acid and 70% TDW. The precipitate was dissolved in ACN-TDW 1:1 and lyophilized. The products were purified by preparative HPLC, and analyzed by MS and analytical HPLC, their degree of purity was determined, and they were sent for biological screening.

NMR Experiments

NMR Measurements and Structure Generation. The sample in lyophilized form was dissolved in 10% deuterium oxide (Aldrich Chemicals Co., USA) in triple distilled water to give a concentration of 3.65 m*M*. The observed pH was 2.2. The NMR experiments were performed on a Bruker Avance 600 MHz DMX spectrometer operating at the proton frequency of 600.13 MHz using a 5 mm selective probe equipped with a self-shielded xyz-gradient coil. A range of temperatures between 2°C and 35°C was examined to find optimal conditions for the NMR measurements. Structural data were acquired at 2°C to reduce molecular mobility. The transmitter frequency was set on the HDO signal, which was calibrated at 4.90 ppm. The residual water resonance was suppressed using a Watergate sequence[40] for TOSCY,[41] COSY[42,43] and preliminary NOESY[44] experiments. TOCSY spectra were recorded using the MLEV-17 pulse scheme for the spin lock at mixing periods of 45–75 ms with 80 scans per t_1 increment.[41] Preliminary NOESY experiments were collected at mixing times of 75, 150, and 200 ms and final structural data were acquired using continuous wave presaturation of the water and with a mixing time of 150 ms and 224 transients for each t_1.

Spectra were processed and analyzed with the XWINNMR and Viewer software packages (Bruker Analytische Messtechnik GmbH) and SPARKY (provided by Goddard T. D. and Kneller D. G., SPARKY 3, University of California, San Francisco). Zero filling in the t_1 dimension and data apodization with a shifted squared sine bell window function in both dimensions were applied before Fourier transformation. The baseline was further corrected in the F_2 dimension with a quadratic polynomial function.

Resonance assignment was done according to the sequential assignment methodology developed by Wüthrich[46] based on the TOCSY, COSY, and NOESY spectra measured under identical experimental conditions. The volumes of the NOE peaks were calculated by SPARKY and translated into distance restraints according to the fixed distance of the well-resolved ortho–para hydrogens of Phe 4. An error of −1.0/+0.5 Å was allowed for all distances. The three-dimensional structures of the peptides were generated using XPLOR (version 3.856).[47] The structures were calculated by hybrid distance geometry-dynamical simulated annealing using XPLOR (version 3.856).[48] The bridge unit residue parameters were generated using XPLO2D (version 2.1)[49] with manual modifications for the protons and by adding appropriate geometry. The bridge unit was introduced using patches within XPLOR, which attached and modified the peptide and bridge to create the final cyclic peptide based on molecular properties within XPLOR. Partial charges were introduced using Gasteiger-Marsili charges[50] and the Amber forcefield[51] by VEGA,[52] using neutral Asp 1 and a charge of −1 on the phosphates. Fifty initial structures were generated by simulated annealing. The NOE energy was introduced as a square-well potential with a constant force constant of 50 kcal/mol Å^2. Simulated annealing consisted of 1500 3-fs steps at 727°C and 3000 1-fs steps during cooling to 27°C. Finally, the structures were minimized using conjugate gradient energy minimization for 4000 iterations. Molmol[45] was used for visual analysis and to analyze the secondary structures of the calculated conformations. Low energy minimized structures chosen for further analysis had no NOE violations, deviations from ideal bond lengths of less than 0.05 Å, and bond angle deviations from ideality of less than 5°.

Electrostatic free energies were derived from finite difference solutions of the Poisson-Boltzman equation using the DelPhi program.[53] The AMBER forcefield[50] was employed and a full Coulombic calculation was performed. The ionic strength of the solution was 10 m*M*, the external dielectric value 80 and the internal 5.0. The ±3 positive and negative isopotential surfaces were presented using CHIMERA.[54]

Biological Assay

Substrate. 35S-radiolabeled IκBα was incorporated into the cellular NF-κB complex: 7 μl of an *in-vitro* translated hemagglutinin-

tagged IκBα[21] were incubated with 0.1 mg Hela lysate prepared as described previously[21] in buffer A [50 m*M*, pH 7.6, 1 m*M* dithiothreitol (DTT)] containing 150 m*M* NaCl 0.1% NP-40 with 4 μl anti NF-κB p65 beads (Santa Cruz SC-109 AC). The slurry was agitated for 2 h at 4°C. The immune complexes were washed extensively with buffer B (1*M* KCl, 0.5% NP-40, 50 m*M* Tris buffer, pH 7.6, 1 m*M* DTT) and buffer A and incubated with constitutively active recombinant IκB kinase (IKK-β), IKK2-EE,[55] in the presence of 2 m*M* adenosine triphosphate (ATP) for 30 min at 37°C. The beads were washed with buffer A.

Ubiquitin Conjugation Assay. The immobilized substrate was agitated for 90 min at 37°C in reaction mixture containing buffer A, 2 m*M* $MgCl_2$, 20 n*M* Okadaic acid, 1 mg/ml bovine ubiquitin (Sigma) and 5 m*M* ATP γS (Sigma), purified E1, recombinant UBC5C and the recombinant E3 $SCF^{\beta\text{-TrCP}}$. The E3 complex was produced and purified from SF9 cells through baculovirus expression of the human SCF proteins β-TrCP, Skp1, Cul1, and Roc1. The beads were boiled in sodium dodecyl sulphate (SDS)-buffer and the sample was separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The gel was analyzed with a phospho-imager (Fujifilm).

Conjugation Inhibition Assay. Peptides were added to the conjugation reaction mixture and their effect on the density of the conjugated substrate band in the gel was measured.

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