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Synthesis and helical structure of lactam bridged BH3 peptides derived from pro-apoptotic Bcl-2 family proteins

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Abstract—Protein–protein interactions within the Bcl-2 family are mediated by the helical BH3 domains of pro-apoptotic family members. To study the mechanism of this BH3 domain–protein interaction, a series of cyclic lactam bridged BH3 peptide analogues were synthesized by a novel combined Fmoc/*t*Bu/Bzl protections strategy. These peptide analogues were studied by circular dichroism spectroscopy and found to adopt highly helical structure. These helical peptides stabilized by a lactam bridge serve as useful models to analyze the structure–function relationship of the pro-apoptotic BH3 domains. Furthermore, the synthetic method for lactam bridge incorporation reported here may find application in studies of other helical structures and development of helix mimics.

The chemical biology of apoptosis or programmed cell death is a rapidly growing area of research that aims to understand the chemical basis of how cells die and develop therapeutic strategies for diseases caused by or related to abnormal cell death mechanisms.[1] A large number of proteins and complex protein–protein interactions are involved in the apoptotic processes. Among these, Bcl-2 and related Bcl-2 family member proteins have been the subject of intensive clinical, biological, structural and chemical research. The Bcl-2 family includes a growing list of proteins that can either inhibit apoptosis (anti-apoptotic) such as Bcl-2 and Bcl-x_L or promote apoptosis (pro-apoptotic) such as Bak, Bax, Bid and Bad. Despite the opposite functions of these anti- and pro-apoptotic proteins (or so called 'Yin' and 'Yang' in cell life and death),[1] they share some common features such as the BH1–4 domains conserved in their amino acid sequences. In general, the BH3 domain is referred to as the 'death domain' as it is shared and used by all pro-apoptotic Bcl-2 family members or the 'Yin' as a key element to bind and counteract anti-apoptotic, 'Yang' proteins. The affinity and selectivity of pro-apoptotic BH3 domains in binding to Bcl-2 or other anti-apoptotic proteins correlate with the death inducing activity of these domains. Synthetic peptides and small molecules that mimic these pro-apoptotic BH3 domains have been shown to induce apoptosis of cancer cells, thus acting as a new class of anticancer agents for a wide variety of cancers in which Bcl-2 or related proteins is implicated.[1] From a medicinal chemistry perspective, understanding the mechanism of Bcl-2 binding by BH3 domains and developing high affinity BH3 mimics are of utmost importance.

In this study, we investigated the chemical mechanism of interaction between BH3 peptide and Bcl-2 protein. Previous NMR studies showed that a 16-residue peptide derived from Bak BH3 domain bound to Bcl-x_L in a helical conformation.[2] However, when unbound to the receptor and even in 30% TFE which promotes helical formation, this Bak BH3 peptide only showed low (22%) helical content.[3] When we conducted circular dichroism (CD) studies of this peptide in more physiologically relevant environment such as PBS buffer or lower 10% TFE, it showed little or no helical content (14% or less) and predominantly random structure. Here we wanted to develop highly stable helical peptides as models to analyze the structure–activity relationship of the BH3 domain. Thus, we synthesized a series of Bak BH3 peptides that incorporated a lactam bridge at various positions of the peptide sequence as a way to increase its helical content when in isolation. The lactam

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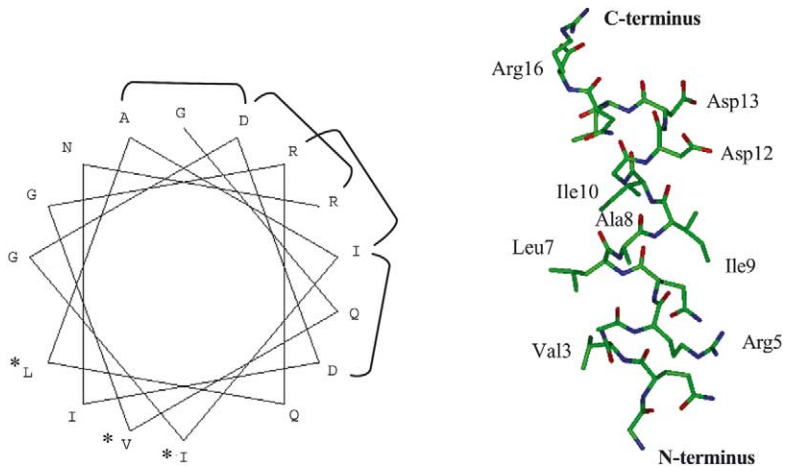


Figure 1. (A) The helical wheel of BakBH3 peptide. The three key Bcl-2 binding residues, Val3, Leu7 and Ile10 are marked by an asterisk. Those residues (or positions for the replacement of appropriate residues) to form the lactam bridge are indicated by the line joining them. (B) The helical structure of BakBH3 peptide. The residues/positions for Bcl-2 binding or lactam bridge formation are shown.

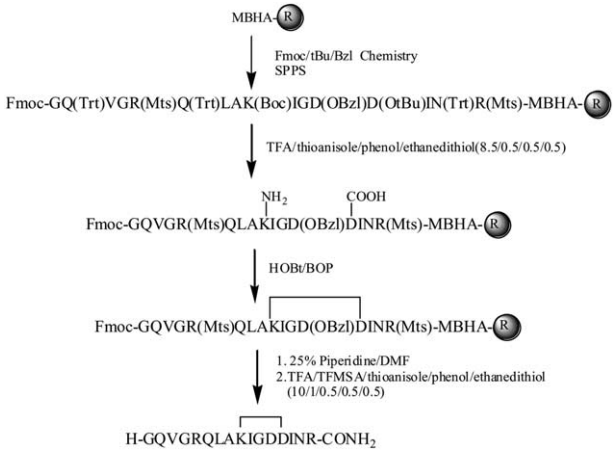
bridge incorporation has been used in a number of studies by other groups to generate highly helical and biologically active peptides.[4–6] Here, we incorporated side chain to side chain i and i+4 lactam bridge into various positions of the 16-residue Bak BH3 peptide, GQVGRQLAIIGDDINR. This parent Bak BH3 peptide has been shown to bind the surface pocket of the Bcl-x_L protein that is essential for its pro-apoptotic activity .[2] Four lactam bridged Bak BH3 peptides, cyclic [Lys,[9] Asp[13]] Bak BH3 (designated as LB1), cyclic [Lys[8], Asp[12]] Bak BH3 (LB2), cyclic [Lys[5], Glu[9]] Bak BH3 (LB3) and cyclic [Asp[12], Lys[16]] Bak BH3 (LB4) (Fig. 1) were synthesized (Table 1).

For the synthesis of lactam bridged cyclic peptides with Fmoc chemistry, the orthogonal side chain protection groups for Lys/Glu or Lys/Asp are generally Dde/Dmab that can be deprotected by hydrazine, Ally/Alloc that can be removed by a Pd-catalyzed reagent, or Mmt/Ppoc that are labile to weak acid. Dde/Dmab is the common choice for the easy removal with hydrazine in DMF although a series of side-products have been reported when Dmab is used to protect the carboxylic acid in Glu.[7] Ally/Alloc have been used for the automated and manual deprotection. However, the Pd-catalyzed reagent [$Pd(PPh_3)_4$] must be handled in the air free environment and requires continuous agitation during the reaction.[8] Mmt/Ppoc protections can be applied to the synthesis of multiple lactam bride containing peptides because of the high sensitivity to acid (1–2%TFA in DCM). Depending on the peptide sequence, several appropriately combined protections such as Fmoc/*t*Bu/cyclohexyl, Fmoc/*t*Bu/allyl,[9] Fmoc/Tos/Boc and Fmoc/Pmc/Dde[10] have been reported for the synthesis of lactam bride peptides. Here in this study, we developed a different combined protection strategy using Fmoc/*t*Bu/Bzl together with MBHA resin. In this method, the side chain carboxylic acid group in Asp or Glu residue destined to form a lactam bridge was protected by O*t*Bu, or otherwise protected by OBzl (Scheme 1). The ε-amino group in Lys destined to form a lactam bridge was protected by Boc, or otherwise protected by benyloxycarbonyl (Z). The TFA/TFMSA two-step deprotection and cleavage procedure (i.e., weak acid to remove the side chain protections first and followed by strong acid to cleave the peptide from the resin) was used in this strategy.[11] MBHA resin was designed for Boc chemistry, but Fmoc chemistry on MBHA resin combined with two-step deprotection and cleavage procedure was reported previously to increase the purity and yield for the synthesis of long and difficult peptide sequences.[12] Here we further developed this strategy for the synthesis of lactam bridged peptides with the combined Fmoc/*t*Bu/Bzl protections. Several advantages of this method

Table 1. Lactam-bridged cyclic BakBH3 peptides

Peptides	Sequences	% Helix (in PBS)
Bak BH3	GQVGRQLAIIGDDINR	14[a]
LB1	GQVGRQLAKIGDDINR	35
LB2	GQVGRQLKIIGDDINR	41
LB3	GQVGKQLAEIGDDINR	78
LB4	GQVGRQLAIIGDDINK	56

[a] In 10% TFE.



Scheme 1. The combined protection scheme of Fmoc/*t*Bu/Bzl for the synthesis of a representative lactam-bridged peptide (LB1).

reported here can be noted. One advantage is that, except for TFA and TFMSA used for the two-step deprotection and cleavage procedure, there are no other special reagents that are required to selectively deblock the side chain protections of the Lys/Asp/Glu residues. Finally, the Fmoc/*t*Bu/Bzl protection strategy allows the direct formation of the lactam bridge on the resin while the peptide assembly proceeds with Fmoc chemistry. Therefore, such a method should be amenable and attractive for the application in combinatorial synthesis.

The secondary structure of these peptide analogues was analyzed by CD spectroscopy.[13] The % α-helical structure as determined by CD demonstrated that all four peptides display α-helical structure in PBS as shown by the two negative bands at 222 nm and 207 nm, and a positive band at 194 nm in CD spectra (Fig. 2). Particularly LB3 and LB4 showed predominant α-helical structure with 78 and 56% helical content, respectively (Table 1). This is in sharp contrast with little or no helical structure in PBS of the parent Bak BH3 peptide or the linear peptides where the lactam bridge is not formed yet (data not shown). It strongly supports the design concept that the cyclic lactam bridge can stabilize and enhance the helical structure of peptides. Bcl-2 binding potency of the lactam bridged BH3 peptides was determined by a fluorescene polarization (FP)-based competitive assay following the protocol published previously.[14] Despite their strong helical structural preference in physiologically mimicking environment, none of these lactam bridged peptides showed any binding to Bcl-2 (data not shown). This result is inconsistent with the previous report by others that the enhanced α-helix in Bad BH3 16-mer peptide increased its binding to Bcl-x_L.[3] There are also reports that the incorporation of lactam brides, despite its enhance of peptide's helical structure, results in reduction in biological activity as in the cases of cationic antimicrobial peptides and human calcitonin.[15,16] Of course, one can not rule out the alternative explanation for the lack of the binding activity of these highly helical peptides that the lactam bridges incorporated might cause steric hindrance with the receptor. Consistent with this possibility are the findings that alanine substitutions at some of the positions that were used for lactam bridge formation in this work (Arg5 in LB3; Ile9 in LB1; and Asp12 in LB2 and LB4) affect the peptide binding to Bcl-x_L.[2] Further studies will be needed to clarify these issues and allow for better understanding of structure–activity relationship of Bcl-2 binding peptides. Nevertheless, the findings from these experiments clearly demonstrated a new method for facile incorporation of a cyclic lactam bridge into peptides with standard solid phase Fmoc chemistry that can induce and stabilize helical structure and be used to study structure–activity relationship of helices (Fig. 3).

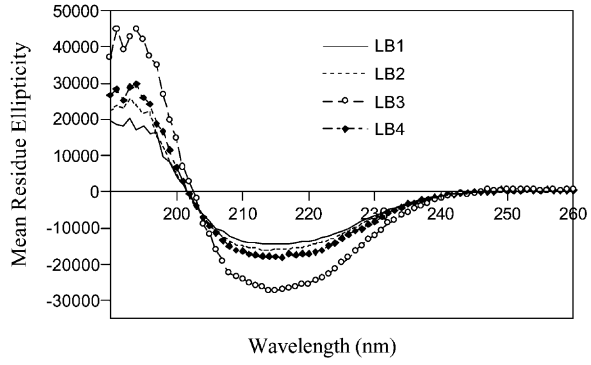


Figure 2. CD spectra of cyclic lactam bridged Bak BH3 peptides.

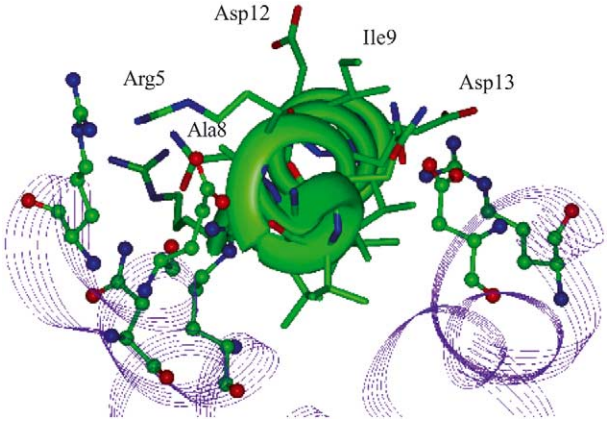


Figure 3. The structural model of BakBH3 peptide completed with Bcl-2 which is built based on the NMR structure of this peptide and Bcl-x_L[2]. The residues or positions that are used for the lactam bridge formation are shown.

Acknowledgements

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References and notes

1. Huang, Z. *Chemistry & Biology* **2002**, *9*, 1059.
2. Sattler, M.; Liang, H.; Nettesheim, D.; Meadows, R. P.; Harlan, J. E.; Eberstadt, M.; Yoon, H. S.; Shuker, S. B.; Chang, B. S.; Minn, A. J.; Thompson, C. B.; Fesik, S. W. *Science* **1997**, *275*, 983.
3. Petros, A. M.; Nettesheim, D. G.; Wang, Y.; Olejniczak, E. T.; Meadows, R. P.; Mack, J.; Swift, K.; Matayoshi, E. D.; Zhang, H.; Thompson, C. B.; Fesik, S. W. *Protein Science* **2000**, *9*, 2528.
4. Felix, A. M.; Heimer, E. P.; Wang, C. T.; Lambros, T. J.; Fournier, A.; Mowles, T. F.; Maines, S.; Campbell, R. M.; Wegrzynski, B. B.; Toome, V.; Fry, D.; Madison, V. S. *J. Pept. Protein Res.* **1988**, *32*, 441.
5. Chorev, M.; Roubini, E.; McKee, R. L.; Gibbons, S. W.; Goldman, M. E.; Caufield, M. P.; Rosenblatt, M. *Biochemistry* **1991**, *30*, 5968.
6. Kapurniotu, A.; Taylor, J. W. *J. Med. Chem.* **1995**, *38*, 836.
7. Johnson, T.; Liley, M.; Cheeseright, T. J.; Begum, F. *J. Chem. Soc., Perkin Trans. 1* **2000**, 2811.
8. Grieco, P.; Gitu, P. M.; Hruby, V. J. *J. Peptide Research* **2001**, *57*, 250.

9. Camarero, J. A.; Cairo, J. J.; Giralt, E.; Andreu, D. *J. Peptide Science* **1995**, *1*, 241.
10. Anichini, B.; Ricci, R.; Fabbri, G.; Balacco, G.; Mauro, S.; Triolo, A.; Altamura, M.; Maggi, C. A.; Quartara, L. *J. Peptide Science* **2000**, *12*, 612.
11. The side chain protecting groups of Fmoc amino acids were as follows: Arg(Mts), Asp(OtBu, or OBzl), Asn(Trt), Glu(O*t*Bu, or OBzl), Gln(Trt) and Lys(Boc or Z). (O*t*Bu, *tert*-butyl ester; Trt, trityl; Boc, *tert*-butyloxycarbonyl; OBzl, benzyl ester; Z, benyloxycarbonyl; Mts, mesitylene-2-sulfonyl). After the peptides were assembled on the 0.1 mmol MBHA resin (0.46 mmol/g, Advanced Chemtech, Louisville, KY), the resins were treated with a mixture consisting of TFA (8.5 mL), thioanisole (0.5 mL), phenol (0.5 mL), and ethanedithiol (0.5 mL) for 30 min $\times 2$. The lactam bridge formations were then performed on resin by BOP/HOBt/DIEA method at least for 24 h. Cyclization reactions were checked using the Kaiser test every 4 h and the further reactions were performed with fresh reagents if the cyclization was incomplete. The cleavages of the lactam bridged peptides were performed by the stand trifluoromethanesulfonic acid (TFMSA) method with a mixture consisting of TFA (10.0 mL), thioanisole (0.5 mL), phenol (0.5 mL), thanedithiol (1.0 mL), ethanedithiol (0.5 mL) and TFMSA (1.0 mL).
12. Yang, B.; Ding, Z.; Han, Z.; Zhang, Q. *Chinese Chemistry Letter* **1999**, *10*, 549.
13. CD measurements were carried out with a JASCO JA-710 spectropolarimeter at 20 °C in 0.1M PBS buffer pH 7.0. CD spectra were recorded over the range of 190–260 nm in a 0.01 cm quartz cuvette (scan speed: 50 nm/s; acquisition time: 1 s). Spectra were signal-averaged four times and buffer-subtracted. CD data was normalized into mean residue ellipticity with formula, $\Theta_{mrd} = \Theta_d \cdot M/(10 \cdot l \cdot c \cdot n_r)$, where l is the pathway length of the curvette, c is the peptide concentration in mg/mL, n_r is the number of residues, M is the molecular weight, Θ_d is the raw CD data. The helical content was estimated using the program CDNN, version 2.1.
14. Wang, J. L.; Liu, D.; Zhang, Z. J.; Shan, S.; Han, X.; Srinivasula, S. M.; Croce, C. M.; Alnemri, E. S.; Huang, Z. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 7124.
15. Houston, M. E., Jr.; Kondejewski, L. H.; Karunaratne, D. N.; Gough, M.; Fidai, S.; Hodges, R. S.; Hancock, R. E. J. J. *Peptide Research* **1998**, *52*, 81.
16. Kapurniotu, A.; Taylor, J. W. *J. Med. Chem.* **1995**, *38*, 836.