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Directed Mutagenesis of the *Bacillus thuringiensis* Cry11A Toxin Reveals a Crucial Role in Larvicidal Activity of Arginine-136 in Helix 4

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Based on the currently proposed toxicity model for the different *Bacillus thuringiensis* Cry δ-endotoxins, their pore-forming activity involves the insertion of the α4-α5 helical hairpin into the membrane of the target midgut epithelial cell. In this study, a number of polar or charged residues in helix 4 within domain I of the 65-kDa dipteran-active Cry11A toxin, Lys-123, Tyr-125, Asn-128, Ser-130, Gln-135, Arg-136, Gln-139 and Glu-141, were initially substituted with alanine by using PCR-based directed mutagenesis. All mutant toxins were expressed as cytoplasmic inclusions in *Escherichia coli* upon induction with IPTG. Similar to the wild-type protoxin inclusion, the solubility of each mutant inclusion in the carbonate buffer, pH 9.0, was relatively low. When *E. coli* cells, expressing each of the mutant proteins, were tested for toxicity against *Aedes aegypti* mosquito-larvae, toxicity was completely abolished for the alanine substitution of arginine at position 136. However, mutations at the other positions still retained a high level of larvicidal activity. Interestingly, further analysis of this critical arginine residue by specific mutagenesis showed that conversions of arginine-136 to aspartate, glutamine, or even to the most conserved residue lysine, also abolished the wild-type activity. The results of this study revealed an important determinant in toxin function for the positively charged side chain of arginine-136 in helix 4 of the Cry11A toxin.

Keywords: *Bacillus thuringiensis*, δ-endotoxin, Inclusion solubility, Larvicidal activity, Site-directed mutagenesis

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Introduction

Bacillus thuringiensis (*Bt*), a Gram-positive endospore-forming bacterium, produces insecticidal proteins in large quantities as different forms of parasporal crystalline inclusions during sporulation (Hofte and Whiteley, 1989). These cytoplasmic inclusions are composed of one or several polypeptides that have been classified as Cry and/or Cyt δ-endotoxins on the basis of the similarity of their deduced amino acid sequences (Hofte and Whiteley, 1989; Crickmore *et al.,* 1998). Currently, the Cry δ-endotoxins have been shown to be active against insect larvae in the orders Diptera (mosquitoes and flies), Lepidoptera (moths and butterflies), Coleoptera (beetles and weevils), and Hymenoptera (wasps and bees) (Schnepf *et al.,* 1998; de Maagd *et al.,* 2001). For instance, the 65-kDa Cry11A toxin and the 130-kDa Cry4B toxin that are produced from *Bt* subsp. *israelensis* are specifically toxic to mosquito larvae (Hofte and Whiteley, 1989; Schnepf *et al.,* 1998).

The *Bt* δ-endotoxins exist as inactive protoxins found within inclusion bodies, which require alkaline solubilisation and proteolytic activation in the insect larval midgut (Hofte and Whiteley, 1989). It has been proposed that, after activation by gut proteases, the active toxins kill the susceptible larvae *via* a two-step receptor mediated mechanism, in which the initial toxin-receptor interaction is followed by membrane insertion of the toxins to form transmembrane leakage pores. These pores cause the target midgut epithelial cells to swell and lyse by colloid-osmotic lysis (Knowles and Ellar, 1987), resulting in extensive damage to the midgut and eventually larval death (Knowles, 1994). However, the precise mechanism of action of the *Bt* toxins is still not completely understood, although knowledge of how these insecticidal proteins work at the molecular level has increased substantially over the last decade.

To date, the three-dimensional structures of two different Cry δ-endotoxins, Cry1Aa, and Cry3A have been solved by

X-ray crystallography (Li, Carroll and Ellar, 1991; Grochulski *et al.*, 1995). Both structures display a high degree of overall structural similarity and are composed of three structurally distinct domains. It is apparent that the N-terminal domain, a seven-helix bundle (six amphipathic helices around a central core helix), is clearly equipped for membrane insertion and pore formation (Li, Carroll and Ellar, 1991; Grochulski *et al.*, 1995). This suggestion has been supported by various studies demonstrating that the isolated helical fragment from different Cry toxins is responsible for pore-forming activity (Walters *et al.*, 1993; Von Tersch *et al.*, 1994; Puntheeranurak *et al.*, 2001).

The molecular mechanism of membrane insertion and pore formation of the Cry toxins is now described in an 'umbrella' model (Knowles, 1994). In this model, α4 and α5 form a helical hairpin to initiate membrane penetration upon specific receptor-binding in which structural rearrangement of the toxin occurs. After insertion of this hairpin, the other helices spread over the membrane surface followed by oligomerization of the toxin (Gazit *et al.*, 1998; Guereca and Bravo, 1999), resulting in formation of an initial tetrameric pore (Schwartz *et al.*, 1997). Currently, this model is supported by a number of experiments, which demonstrates the crucial role of α4 and α5 in pore-forming activity of different Cry toxins (Schwartz *et al.*, 1997; Kumar and Aronson, 1999; Masson *et al.*, 1999; Nunes-Valdes *et al.*, 2001). Recent studies clearly demonstrated that the helix 4-loop-helix 5 hairpin is more active in membrane penetration than each of the isolated helices, or their mixtures, consistent with its function as the membrane-inserted portion of the Cry toxins (Gerber and Shai, 2000).

In earlier studies, we demonstrated that α4 and α5 of the 130-kDa Cry4B toxin are essential determinants of toxicity, likely to be involved in pore formation rather than in receptor recognition (Uawithya *et al.*, 1998; Sramala *et al.*, 2000). In addition, arginine-158 in helix 4 was found to play an important role in larvicidal activity of this toxin (Sramala *et al.*, 2001). In the present report, an analogous effect on toxicity was observed for the 65-kDa dipteran-specific Cry11A toxin when charged, and polar residues in helix 4 were altered. The results revealed that the specific structure for the positively charged side chain of arginine-136 in this helix is directly involved in Cry11A toxin activity, supporting the notion that α4 is essential for pore formation by the Cry δ-endotoxins.

Materials and Methods

Plamids and site-directed mutagenesis The full-length gene, encoding the 65-kDa Cry11A toxin from the recombinant plasmid pBTC68A (a generous gift of Dr. Wattanalai Panbangred, Department of Biotechnology, Mahidol University, Thailand), was subcloned into the pMEx8 expression vector (Buttcher *et al.*, 1990). This resultant plasmid (pME4D) was used as a template for site-directed mutagenesis. Each complementary pair of mutagenic oligonucleotide primers was purchased from Genset Inc. (Singapore), as shown in Table 1. All mutations were generated by PCR using high fidelity *Pfu* DNA polymerase following the procedure of the QuickChange™ mutagenesis kit (Stratagene). All mutant plasmids were analyzed by DNA sequencing using a Perkin Elmer ABI prism 377 automated sequencer.

Table 1. Complementary primers for substituting a coded residue with different amino acids.

Primer	Sequence[a]	Restriction Site
	A T A A G Y F L N	
K123A-f	5'CTGCAACAGCTGCAGGTTATTTCTAAATC 3'	*Pst*I
K123A-r	5'GATTTAGAAAATAACCTGCAGCTGTTGCAG 3'	
	G A I I Q A L P Q F	
R136A-f	5'GTGGTGCTATAATACAAGCTTTACCTCAATTTG 3'	*Hind*III
R136A-r	5'CAAATTGAGGTAAAGCTTGTATTATAGCACCAC 3'	
	I Q R L P Q F A V Q T	
E141A-f	5'TAATACAACGTCTACCTCAATTTGCAGTTCAAACAT 3'	*Acc*I
E141A-r	5'ATGTTTGAACTGCAAATTGAGGTAGACGTTGTATTA 3'	
	L N L S G A I I Q K L P	
R136K-f	5'CTAAATCTAAGTGGGGCCATAATACAAAAATTACCTC 3'	*Hae*III
R136K-r	5'GAGGTAATTTTTGTATTATGGCCCCACTTAGATTTAG 3'	
	L N L S G A I I Q Q L P	
R136Q-f	5'CTAAATCTAAGTGGTGCCATAATACAACAATTACCTC 3'	*Nla*IV
R136Q-r	5'GAGGTAATTGTTGTATTATGGCACCACTTAGATTTAG 3'	
	G A I I Q D L P Q F E	
R136D-f	5'GGTGCTATAATACAAGATCTACCTCAATTTGAGG 3'	*Bgl*II
R136D-r	5'CCTCAAATTGAGGTAGATCTTGTATTATAGCACC 3'	
	L S G A I I A R L P Q F	
Q135A-f	5'CTAAGTGGTGCCATAATAGCTAGGTTACCTCAATTTG 3'	*Bst*EII & *Nla*IV
Q135A-r	5'CAAATTGAGGTAACCTAGCTATTATGGCACCACTTAG 3'	
	A I I Q R L P A F E V	
Q139A-f	5'GCTATAATACAACGTCTACCTGCTTTTGAGGTTC 3'	*Acc*I
Q139A-r	5'GAACCTCAAAAGCAGGTAGACGTTGTATTATAGC 3'	
	T A K G A F L N L	
Y125A-f	5'CAACAGCCAAGGGTGCCTTTCTAAATCTAAG 3'	*Nla*IV
Y125A-r	5'CTTAGATTTAGAAAGGCACCCTTGGCTGTTG 3'	
	G Y F L A L S G	
N128A-f	5'GGGTTATTTTCTAGCATTAAGTGGTGC 3'	*Dde*I
N128A-r	5'GCACCACTTAATGCTAGAAAATAACCC 3'	
	Y F L N L A G A I I	
S130A-f	5'GTTATTTTCTAAATCTGGCCGGTGCTATAATAC 3'	*Hae*III
S130A-r	5'GTATTATAGCACCGGCCAGATTTAGAAAATAAC 3'	

[a]Introduced restriction enzyme recognition sites are underlined. The mutated nucleotide residues are shown as boldface. Deduced amino acid sequences are shown on top of each pair of oligonucleotide primers.

Toxin expression and characterization The wild type and mutant Cry11A toxin genes were expressed in the *E.coli* strain JM109 under control of the inducible *tac* promoter. Cells were grown in a LB medium that was supplemented with 100 μg ml^{-1} ampicillin until OD_{600} reached 0.4-0.5. Incubation was continued for another 4 h after addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the final concentration of 0.1 mM. Protein expression was analyzed by sodium dodecyl sulfate (SDS)-15% w/v polyacrylamide gel electrophoresis (PAGE). Immunoblotting was performed with polyclonal rabbit antibodies against the Cry11A toxin (kindly provided by Prof. David Ellar, University of Cambridge, UK). Immunocomplexes were detected with an anti-rabbit antibody-alkaline phosphatase conjugate (Sigma).

E. coli cultures, expressing each mutant as cytoplasmic inclusion bodies, were harvested by centrifugation, resuspended in distilled

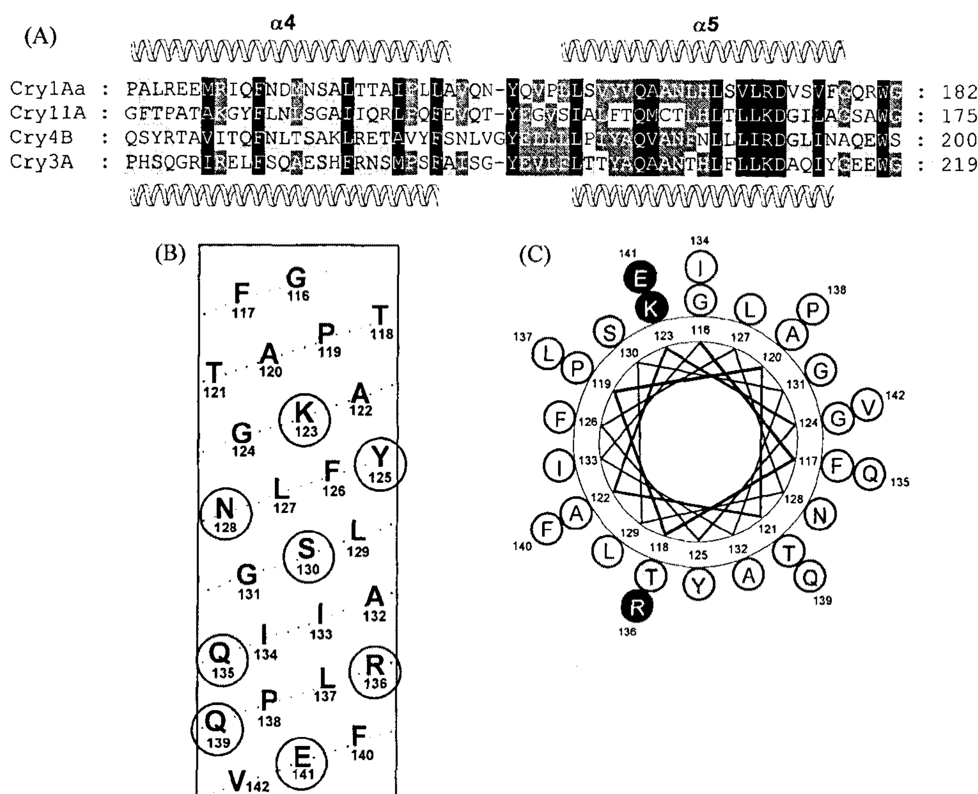

Fig. 1. (A) Multiple sequence alignment of helices 4 and 5 of Cry11A with the crystal structures of Cry1Aa and Cry3A, and the homology-based model of Cry4B. The sequences were aligned using the program CLUSTAL W. The degree of conservation is represented by background shading of the residues with the darkest shading for the most conserved: 100% conserved, 75% conserved, and 50% conserved. The positions of secondary structure elements of Cry1Aa and Cry3A are illustrated over and under the alignments, respectively. (B) The predicted pattern of helix 4 of Cry11A is composed of 27 residues of which the encircled residues were mutated. (C) A helical wheel projection of helix 4 of Cry11A. Amino acid residues are plotted every 100 degrees consecutively around the wheel, following the sequences given in B. The following color code is used: black is an amino acid with a charged side chain, gray is a polar side chain, and white is a hydrophobic side chain.

water, and disrupted in a French Pressure Cell at 16,000 psi. The crude lysates were centrifuged at 8,000 g for 5 min and the pellets obtained were washed 3 times in distilled water. Protein concentrations were determined by using a protein microassay (Bio-Rad) with the bovine serum albumin fraction V (Sigma) as a standard. Protoxin inclusions (1 mg ml^{-1}) were solubilized in 50 mM Na_2CO_3, pH 9.0 and incubated at 37°C for 60 min, as described previously (Uawithya *et al.*, 1998). After centrifugation for 10 min, the supernatants were analyzed by SDS-PAGE in comparison with the inclusion suspension.

Larvicidal activity assays Bioassays were performed, as described previously (Angsuthanasombat *et al.*, 1993), using 2-day old *Aedes aegypti* mosquito-larvae reared from eggs that were supplied by the mosquito-rearing facility of the Institute of Molecular Biology and Genetics, Mahidol University, Thailand. About 500 larvae were reared in a container (22 × 30 × 10 cm) with approximately 3 litres of distilled water that was supplemented with a small amount of rat diet pellets. Both rearing and bioassays were performed at room temperature (25°C). The assays were carried out in 1 ml of *E. coli* suspension (10^8 cells suspended in distilled water) in a 48-well micrometer plate (11.3 mm well diameter) with 10 larvae per well and a total of 100 larvae for each type of *E. coli* samples. *E. coli* cells, containing the recombinant plasmid pME4D and the pMEx8 vector, were used as positive and negative controls, respectively. Mortality was recorded after a 24-hour incubation period.

Results and Discussion

Based on a multiple-amino acid sequence alignment with the known crystal structures of Cry1Aa and Cry3A (Li, Carroll, and Ellar, 1991; Grochulski *et al.*, 1995), and the homology-based model of Cry4B (Uawithya *et al.*, 1999), the predicted α4 and α5 were located within the pore-forming domain of Cry11A (see Fig. 1A). Charged amino acids in helix 4 were shown to be critical for toxin activity (Kumar and Aronson, 1999; Masson *et al.*, 1999; Sramala *et al.*, 2001). To investigate the possible role for toxicity of charged and polar amino acids in α4 of Cry11A, a PCR-based mutagenesis strategy, previously employed for Cry4B (Sramala *et al.*, 2001), was applied to obtain substitutions within Cry11A. We initially generated eight Cry11A mutants in which three

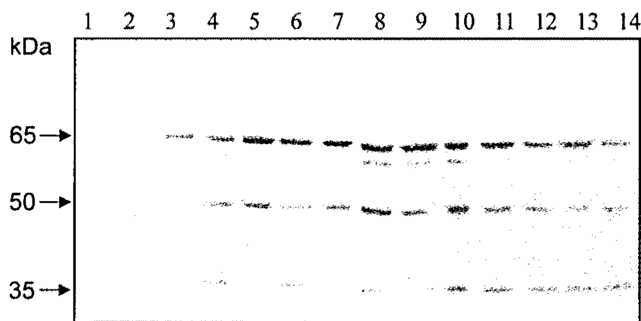

Fig. 2. Western blot analysis of lysates extracted from *E. coli* cells harboring the pMEx8 vector (lane 2), pME4D (lane 3), or the mutant plasmids-K123A, Y125A, N128A, S130A, Q135A, R136A, R136K, R136Q, R136D, Q139A, and E141A (lanes 4-14, respectively)-showing the 65-kDa Cry11 toxin and small molecular fragments that cross-reacted with the Cry11A antibodies. Lane 1 represents the molecular mass standards.

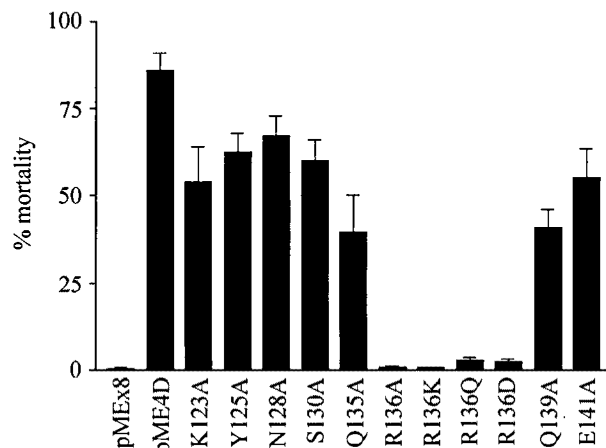

Fig. 3. Mosquito-larvicidal activities of *E. coli* cells expressing the Cry11A wild-type toxin (pME4D), or its mutants-K123A, Y125A, N128A, S130A, Q135A, R136A, R136K, R136Q, R136D, Q139A and E141A)-against *Aedes aegypti* larvae. Error bars indicate standard error of the mean from the three independent experiments.

charged and five polar amino acids in helix 4 (Fig. 1B) were substituted with alanine. Most of the targeted residues, Tyr-125, Asn-128, Gln-135, Arg-136 and Gln-139, but not Lys-123, Ser-130 and Glu-141, are located at the hydrophilic surface (see Fig. 1C).

Expression of the mutant toxins in *E. coli* was controlled by the *tac* promoter. Upon addition of IPTG to mid-exponential phase cultures, all mutant toxins were predominantly produced as sedimentable inclusion bodies. Lysates were analyzed by SDS-PAGE and immunoblotting, and the protein expression level of all mutant derivatives was found to be comparable to the wild type. The 65-kDa expressed mutant proteins specifically cross-reacted with antibodies raised against the Cry11A toxin (see Fig. 2). However, two relatively intense immuno-reactive bands of ca. 50 kDa and ca. 35 kDa were detected in all mutant lysates. This indicates that the expressed mutant proteins are rather sensitive to proteolytic degradation.

The solubility of mutant protein inclusions in comparison to the wild-type inclusion was assessed using a carbonate buffer, pH 9.0. The amount of 65-kDa soluble proteins in the supernatant was compared with those of the proteins initially used, in order to determine the percentage of protein solubilisation. All of the mutant inclusions were found to be soluble to some extent in this buffer, giving less than 20% solubility, which resembles closely the wild-type inclusions under similar conditions.

To determine the effect of mutations on toxicity, *E. coli* cells that expressed each type of the mutant toxin were tested for their relative biological activity towards *Aedes aegypti* larvae. All of the assays were carried out in ten replicas for each sample and repeated three times; the mortality data recorded after a 24-hour incubation are shown in Fig. 3. Interestingly, only the R136A mutation resulted in a total loss of larvicidal activity, while alanine substitutions at seven other positions (K123A, Y125A, N128A, S130A, Q135A, Q139A and E141A) still retained over 50% of the wild-type activity. When this critical arginine residue at position 136 was converted to aspartate, glutamine or even to the most conserved residue for positively charged side chain, *i.e.* lysine, all R136 mutants (R136D, R136Q and R136K) were shown to be nontoxic to mosquito larvae (see Fig. 3). These results could imply the requirement for a specific structure of the positive side chain at this position. Perhaps Arg-136, which is likely to face the pore lumen, could interact with an aqueous environment, and somehow stabilize the functional pore. However, the precise function of this residue remains to be elucidated.

Protein expression levels and solubility of the inclusions suggested that the complete loss of toxicity observed for the R136A mutant is least likely to be caused by misfolding of the protein. Taken together, our results indicate that Arg-136 is a critical residue involved in Cry11A toxin activity. The data further support our previous findings that Arg-158 in α4 played a crucial role in toxicity of the 130-kDa Cry4B toxin, since the single alanine substitution at this residue almost completely abolished its activity towards mosquito larvae (Sramala *et al.*, 2001). In addition, results reported by other workers revealed that an arginine residue at position 131 in α4 is important for toxicity of both the lepidopteran-specific Cry1Aa and Cry1Ac toxins (Kumar and Aronson, 1999; Masson *et al.*, 1999). Two other negatively charged residues (Glu-129 and Asp-136) of Cry1Aa were also shown to be critical in the passage of ions through the pore (Masson *et al.*, 1999).

Comparisons of structural models among Cry11A, Cry4B, and Cry1Aa suggest that, although Arg-136 of Cry11A is located on the opposite side of helix 4 relative to Arg-158 of Cry4B or Arg-131 of Cry1Aa, all of these three critical

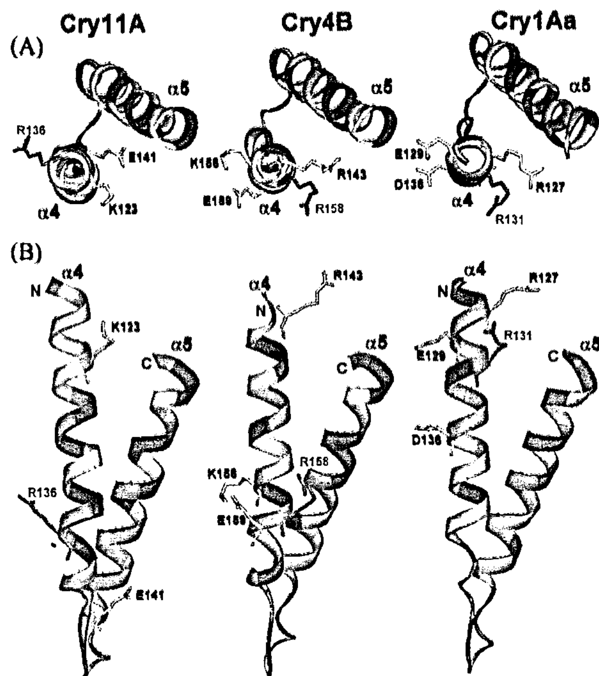

Fig. 4. (A) Top and (B) side views of amino acid arrangement in helix 4 together with the relative position of helix 5 in 3D models of Cry11A and Cry4B built by homology modeling and the Cry1Aa crystal structure. The labeled residues, shown in red and yellow, indicate the critical arginine residues and other charged positions, respectively. The structures were prepared using Weblab viewer (Molecular Simulations Inc.).

residues are oriented on the side of helix 4, which is furthest away from helix 5 (see Fig. 4A). It should be noted that Arg-136 and Arg-158, in both of the dipteran-specific toxins, are situated near the C-terminal end of helix 4, while Arg-131 of the lepidopteran-specific Cry1Aa toxin is located furthest from the C-terminus of this helix (Fig. 4B). Differences in the location of these critical residues may conceivably reflect the diversity in the channel architecture for each group of insect-specific Cry toxins. Further studies are required to elucidate the role of these positively charged residues in helix 4, to discover whether they are involved in the passage of ions through the pore.

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