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Cobra venom contains a pool of cysteine-rich secretory proteins

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Abstract

A large family of cysteine-rich secretory proteins (CRISPs) includes proteins of different origin, the function of the majority of CRISPs being unknown. For CRISPs isolated from snake venom, two types of activities were found: two proteins blocked cyclic nucleotide-gated ion channels, several others blocked potassium-stimulated smooth muscle contraction. Thus, snake CRISPs represent potentially valuable tools for studies of ion channels, which makes promising a search for new CRISPs. Here we report on the isolation of several novel CRISPs from the venoms of Asian cobra *Naja kaouthia* and African cobra *Naja haje* using a combination of different types of liquid chromatography. Four CRISP variants were identified in *N. kaouthia* venom and three proteins, one of them acidic, were found in *N. haje* venom. Acidic CRISP was found in a reptilian venom for the first time. Our data suggest that each cobra venom contains a pool of different CRISPs.

Keywords: Cobra venom; Cysteine-rich secretory proteins; Isolation; Properties

A family of cysteine-rich secretory proteins (CRISPs) is a group of secreted single-chain proteins with molecular weights of about 20–30 kDa and homologous amino acid sequences comprising 16 conserved cysteine residues which form 8 disulfide bonds. Fourteen cysteine residues are located within the C-terminal half, 10 of them being within the C-terminal third of the polypeptide chain. However, the N-terminus is more conserved as compared to other regions of these proteins.

CRISPs have been found in secretions of mammalian exocrine glands, first of all in male reproductive tracts [1], in human granulocytes and plasma [2], and in some organisms of other taxons (for references see [3]), including reptilians. The reptilian CRISPs were found mainly in their venoms.

The first described reptilian CRISP was helothermine from the skin secret of the lizard *Heloderma horridum horridum* [4]. Later on a number of CRISPs were isolated from the venoms of snakes belonging to different families and inhabiting different continents. Thus, CRVP (cysteine-rich venom protein) from *Trimeresurus mucrosquamatus* (*Viperidae*, Asia) [5], pseudechetoxin from *Pseudechis australis* (*Elapidae*, Australia) [6], protein 25k from *Naja kaouthia* (*Elapidae*, Asia) [7], latisemin from sea snake *Laticauda semifasciata* (*Hydrophidae*), and tigrin from *Rhabdophis tigrinus tigrinus* (*Colubridae*) [8] have been described. Recently, the list of Asian Elapid CRISPs has been extended by ophanin from *Ophiophagus hannah* [9], bucarin from *Bungarus candidus* (Accession #P81993 in SwissProt), and by two proteins from *Naja atra*, CRVP1 and CRVP2 [10]. Protein 25k and CRVP1 represent probably one and the same protein as sequences of protein 25k fragments comprising 101 residues, determined by peptide sequencing, coincide completely with corresponding fragments of CRVP1 amino acid sequence deduced from cDNA.

All these snake venom proteins consist of a single polypeptide chain with a molecular mass of 23–26 kDa. They are basic in nature and contain 8 disulfide bonds.

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The list of Elapid CRISPs seems to be far from completion. Thus, cross-reactivity screening of *Elapidae* venoms with antiserum to triflin, a CRISP from the venom of *Trimeresurus flavoviridis* (*Viperidae*), detected the presence of similar antigens in venoms of *Hemachatus hemachatus*, *Naja mocanbigue*, *Naja nivea*, and of two antigens in *N. haje* venom [9]. Yamazaki and Morita [3] communicated on the presence of two CRISPs in *N. kaouthia* venom, but did not give any details on them. Here we report on the isolation of two novel CRISPs from the cobra *N. kaouthia* venom and three CRISPs from the African cobra *N. haje* venom, one of the *N. haje* proteins being acidic.

Materials and methods

Naja kaouthia and *Naja haje* venoms were obtained from living cobras kept in captivity as described in [11]. All other reagents were of the highest purity commercially available.

Isolation and purification of proteins

Eight hundred milligrams of dried venom was dissolved in 1 ml of 0.1 M ammonium acetate buffer, pH 6.2, and applied onto a column (4.5 × 150 cm) with Sephadex G50 sf (Amersham Biosciences) equilibrated with the same buffer. Fractions I–IV were pooled as depicted in Figs. 1A and B and lyophilized. Fraction II *N. kaouthia* was separated on a HEMA BIO 1000 CM column (8 × 250 mm, Tessek, Czech Republic) in an ammonium acetate gradient from 5 to 600 mM (pH 7.5) in 60 min at a flow rate of 1.4 ml/min. Separation of fraction II *N. haje* was performed on the same column with the following program: isocratic elution in 5 mM ammonium acetate (pH 7.5) for 5 min, then linear gradient from 5 to 140 mM in 14 min and from 140 to 180 mM in 20 min, followed by two more gradients: from 180 to 360 mM in 11 min and from 360 to 400 mM in 20 min, and final isocratic elution in 400 mM ammonium acetate for 5 min.

Fractions II-6, II-10, and II-11 of *N. kaouthia* venom as well as II-4 and II-7 of *N. haje* venom were lyophilized and further separated on a Vydac C18 column (4.6 × 250 mm) in an acetonitrile gradient from 15 to 45% in 30 min in the presence of 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. Fraction II-1 of *N. haje* venom from the cation-exchange column was separated on a HEMA BIO1000 DEAE column (8 × 250 mm, Tessek, Czech Republic) in an ammonium acetate gradient from 5 to 400 mM (pH 7.5) in 40 min at a flow rate of 0.5 ml/min.

Electrophoresis in 12% polyacrylamide gel in the presence of sodium dodecyl sulfate (PAGE–SDS) both under reducing and non-reducing conditions was performed according to [12].

Pyridylethylation, Edman degradation, and recording of matrix-assisted laser desorption ionization (MALDI) mass spectra were performed as described earlier [11]. An alignment and comparison of determined N-terminal amino acid sequences to those of known proteins were performed using program BLASTP 2.2.5 at ExPASy molecular biology server ([13]; http://us.expasy.org/cgi-bin/blast.pl).

Fluorescence spectra were recorded in 50 mM Tris–HCl buffer, pH 7.4, on Hitachi F-4000 spectrofluorimeter.

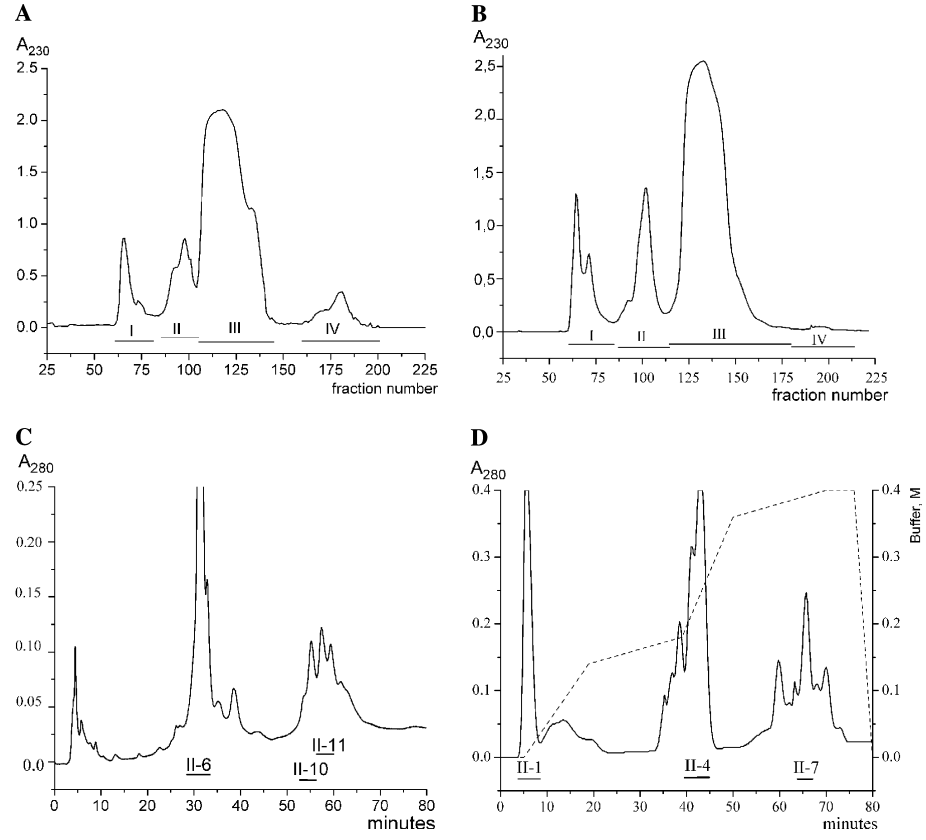

Fig. 1. Separation of crude venoms of *N. kaouthia* (A) and *N. haje* (B) by gel filtration on Sephadex G-50sf column (4.5 × 150 cm) in 0.1 M ammonium acetate buffer, pH 6.2, fraction volume 9 ml. Separation of fractions II of *N. kaouthia* (C) and *N. haje* (D) by cation-exchange HPLC on a HEMA BIO 1000 CM column in a gradient of ammonium acetate concentration (pH 7.5).

In vivo toxicity study

All the appropriate actions were taken to minimize discomfort to mice. World Health Organization's International Guiding Principles for Biomedical Research involving Animals were followed during experiments on animals. Solutions of proteins purified were administered intraabdominally to white autobred line NMRI mice ($n = 3$) and to cockroaches ($n = 5$) at doses up to 4 nmol/g as well as to crickets ($n = 5$) at doses up to 5 nmol/g [14].

Results and discussion

To obtain CRISPs from cobra venoms we applied a separation scheme used earlier for isolation of different venom components [11]. Gel-filtration on Sephadex G-50 sf was used as the first step (Figs. 1A and B). By this procedure fraction II containing proteins with molecular masses in the range from 9 to 30 kDa was separated from fraction III containing highly toxic proteins (cytotoxins, α-neurotoxins, etc.) with masses of 6–8 kDa. Fractions II were further separated on a cation-exchange column. Sub-fractions obtained were analyzed by SDS–PAGE. Single-chain proteins with masses of 23–25 kDa were detected in sub-fractions II-6, II-10, and II-11 of *N. kaouthia* venom and in sub-fractions II-1, II-4, and II-7 of *N. haje* venom (Figs. 1C and D). Individual proteins were isolated from these sub-fractions by reversed-phase HPLC (Figs. 2A and B), the exception was sub-fraction II-1 *N. haje* which contained acidic proteins and was separated by anion-exchange HPLC (Fig. 2C). Thus, at least three proteins with a molecular masses in the range from 23 to 25 kDa were found in each of the venoms. Molecular masses of the individual compounds were more precisely determined by MALDI mass spectrometry (Table 1) and the proteins were named according to their masses. It should be noted that protein with molecular mass of 24,953 Da and N-terminal sequence similar to that of CRVP-25k was found in cobra *Naja oxiana* venom. Interestingly, two peaks of comparable intensity were detected in the MALDI mass spectra of protein CRVP-23k (23,621 and 23,704 Da) and protein CRVP-24k (24,080 and 24,179 Da).

The yield of CRVP-25 h was about 0.5%, close to that of CRVP-25k. The yields of the other CRISPs (CRVP-23k, CRVP-24k, CRVP-23h, and CRVP-25h-A) were about 0.1–0.2%.

For structural characterization, pyridylethylated derivatives of the isolated proteins were obtained and analyzed by MALDI mass spectrometry. Differences between masses of the intact proteins and their derivatives were about 1.60–1.65 kDa indicating the presence of 16 cysteine residues in a polypeptide chain. This is the main feature of CRISPs. The established N-terminal sequences are given in table along with the closest homologue sequences. These data clearly indicate that all the isolated proteins belong to the CRISP family.

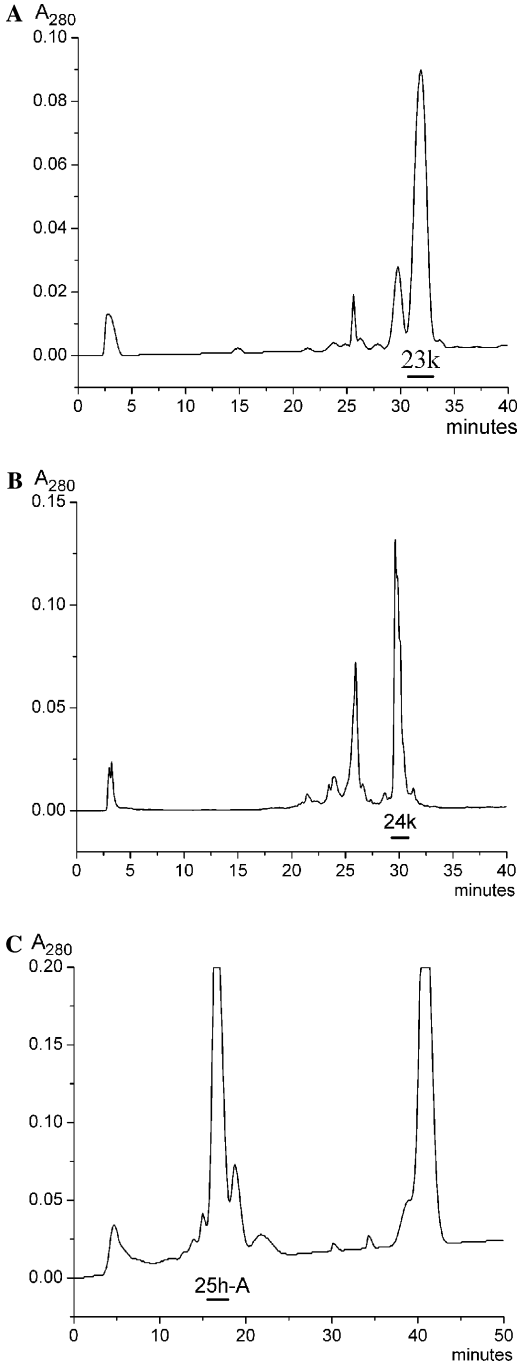

Fig. 2. Purification of proteins CRVP-23k and CRVP-24k from *N. kaouthia* fractions II-11 (A) and II-10 (B), respectively, by reversed-phase HPLC on a VYDAC C18 column as well as of CRVP-25h-A (C) from *N. haje* fraction II-1 by anion-exchange HPLC on a HEMA BIO1000 DEAE column.

It is interesting that all snake CRISPs can be classified into three different sub-groups basing on differences in their amino acid sequences (Fig. 3). The first sub-group includes 9 proteins with the longest sequences (positions 1–9 in Fig. 3) consisting of 221 amino acid residues. CRVP-25k (25k), CRVP-25h, and CRVP-25h-A apparently also belong to this sub-group. Thus,

Table 1
Comparison of novel proteins isolated from the *N. kaouthia* and *N. haje* cobra venoms with known snake venom CRISPs

Protein	Species	Fraction number	Molecular mass (Da)	Swiss-Prot Accession #	N-terminal amino acid sequence
CRVP-25k	***N. kaouthia***	II-6 (Fig. 1C)	24,953 (MALDI MS)		NVDFNSESTRRKKKQKEIVDLHNSL
CRVP-25h	***N. haje***	II-4 (Fig. 1D)	24,960 (MALDI MS)		NVDFNSESTRRKKKQKEIVDLXNSL
CRVP-25h-A	***N. haje***	II-1 (Fig. 1D)	25,006 (MALDI MS)		DVDFNSESTRRKNKQKEIVDLHNSLKKTV
Ophanin	*O. hannah*		24,960 (cDNA)	Q7ZT98	NVDFNSESTRRQKKQKEIVDLHNSLRRSV
CRVP-1	*N. atra*		24,954 (cDNA)	Q7T1K6	NVDFNSESTRRKKKQKEIVDLHNSLRRRV
Triflin	*T. flavoviridis*		24,798 (cDNA)	Q8JI39	NVDFDSESPRKPEIQNEIIDLHNSLRRSV
CRVP	*T. mucrosquam.*		24,725 (cDNA)	P79845	NVDFDSESPRKPEIQNEIIDLHNSLRRSV
Tigrin	*R. tigrinus*		24,736 (cDNA)	Q8JGT9	NVDFNSESPRNPGKQQEIVNIHNSFRRSV
CRVP-24k[a]	***N. kaouthia***	II-10 (Fig. 1C)	24,080; 24,179 (MALDI MS)		VDFASESXNKRENQ-QIVDKHNAL
CRVP-2	*N. atra*		24,302 (cDNA)	Q7ZZN8	TVDFASESSNKRENQKQIVDKHNALRRSV
Latisemin	*L. semifasciata*		24,272 (cDNA)	Q8JI38	TVDFASESSNKRENQKEIVDKHNALRRSV
CRV1	*L. hardwickii*		24,350 (cDNA)	Q8UW25	TVDFASESSNKKDYRREIVDKHNALRRSV
CRV2	*L. hardwickii*		24,538 (cDNA)	Q8UW11	TVDFASESSNKKDYQREIVDKHNALRRSV
CRVP-23k	***N. kaouthia***	II-11 (Fig. 1C)	23,621; 23704 (MALDI MS)		ESSNKRENQKQIV
CRVP-23h	***N. haje***	II-7 (Fig. 1D)	23,590 (MALDI MS)		DSSNLPPNQKQIVD
Pseudechetoxin[b]	*P. australis*		23,722 (cDNA)	Q8AVA4	SNKKNYQKEIVDKHNALRRSV
Bucarin	*B. candidus*		Not established	P81993	ESSNKRENQKQIVDKHNALRRSV

The new proteins are shown in bold.
[a] Two sequences were determined for CRVP-24k, one of them lacks N-terminal valine.
[b] Homologous pseudecin (Accession No. #Q8AVA3) from *P. porphyriacus* lacks N-terminal serine.

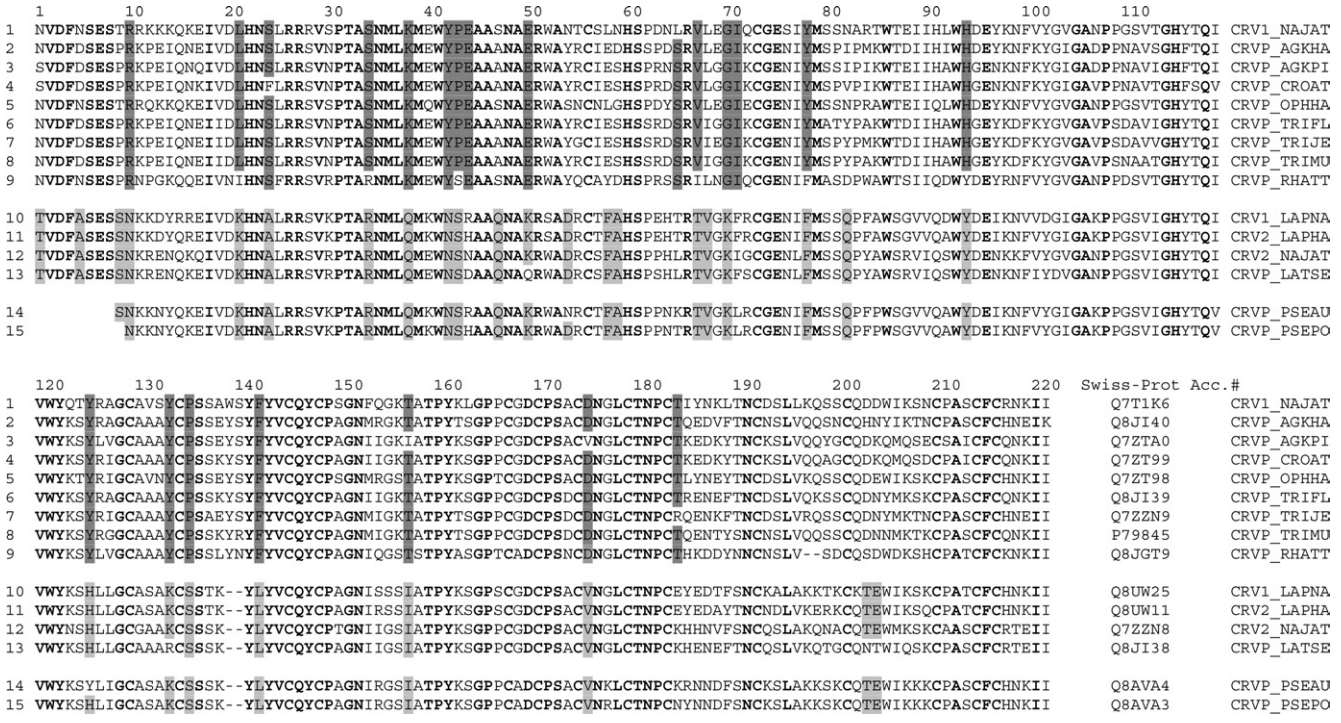

Fig. 3. Known snake venom CRISPs combined according to similarities in their amino acid sequences. Positions 1–9, "long" CRISPs; 10–13, "intermediate;" and 14–15, "short." Amino acid residues invariant in all proteins are shown in bold. The amino acid residues invariant in "long" CRISPs are indicated by dark grey shading and those invariant in "intermediate" and "short" CRISPs are shaded in light grey.

amino acid sequences of 25k fragments [7] coincide completely with those of CRVP1 [10]. N-terminal amino acid sequences of CRVP-25h and CRVP-25h-A, and molecular masses of these proteins have the highest similarity to those of the longest venom CRISPs.

The second sub-group includes the proteins of intermediate length (positions 10–13 in Fig. 3) with amino acid sequences consisting of 219 residues. Their sequences differ clearly from those of the proteins of the previous group, despite the small difference in their

lengths. CRVP-24k can be assigned to this sub-group as its N-terminal sequence has the highest homology to the venom CRISPs from this sub-group and its molecular mass is closer to masses of proteins from this sub-group than from two others.

The last sub-group includes two snake venom CRISPs with the shortest sequences: pseudechetoxin (211 residues) and pseudecin from *Pseudechis porphyriacus* venom (210 residues). CRVP-23k and CRVP-23h are also included in this sub-group as they have truncated N-terminal amino acid sequences and their molecular masses are similar to those of pseudecin and pseudechetoxin.

Notably, the sequences of proteins from "intermediate" group are more similar to those of "short" CRISPs than to "long" CRISPs. The amino acid residues differing in charge or hydrophobicity and being conservative in "long" CRISPs on the one side and in "intermediate" as well as "short" on the other are indicated in Fig. 3 by different shading.

It should be noted that an acidic CRISP (CRVP-25h-A) has been found in a venom for the first time. To compare CRVP-25h-A and a basic protein CRVP-25h, the fluorescence spectra were recorded for both proteins. The emission spectrum of CRVP-25h-A has a maximum at 344 nm and bandwidth at half maximum 56.4 nm, and for CRVP-25h these values are 348.8 nm and 67.5 nm, respectively. These data indicate a similar exposure into a solvent of all tryptophane residues in these proteins.

The most obvious test for biological activity of venom compounds is determination of acute toxicity. Taking in account the low content of the novel CRISPs, the toxicity study was performed for more abundant of them. CRVP-24k was administered to cockroaches, CRVP-25k—to cockroaches and mice, and CRVP-23h, CRVP-25h, and CRVP-25h-A—to crickets. No lethal effect was observed at doses up to 5 nmol/g. Basing on similarity of their physical and chemical properties, one might conclude that all CRISPs isolated from *N. haje* and *N. kaouthia* venoms are non-toxic to both insects and mammals.

The finding of pools of CRISPs in the venoms of the two cobra species raises a question about their role in a damaging action of snake venom. Although the conservation of venom CRISPs can now be traced up through all the families of venomous snakes including Australian, Asian, and African Elapids, evidences for their biological effects are not numerous to date. Thus, pseudechetoxin and pseudecin block cyclic nucleotide-gated ionic channels with different efficiencies [6,15], while protein CRVP-25k from *N. kaouthia* practically does not inhibit these channels [7]. Interestingly, pseudechetoxin and pseudecin on the one hand and CRVP-25k on the other belong to different CRISP sub-groups: "short" and "long," respectively. It was shown [8] that triflin and ophanin from "long" sub-group and latisemin from "intermediate" sub-group inhibit tail vein muscle contraction induced by high concentration of potassium ions. Basing on these data, one can suggest that snake venom CRISPs belonging to different sub-groups act on different biological targets, contributing in this way to diversity of damaging effects of snake venoms.

Our finding of CRISPs belonging to different sub-groups in the venom of two cobra species allows us to suggest that each cobra venom contains a pool of CRISPs that might have different biological activities.

Acknowledgments

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