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Contryphan Is a D-Tryptophan-containing *Conus* Peptide*

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In this report, we document for the first time the occurrence of D-tryptophan in a normally translated polypeptide, contryphan. The peptide, isolated from the venom of the fish-hunting marine snail *Conus radiatus*, produces the "stiff-tail" syndrome in mice. Characterization of the octapeptide gave the following sequence,

Gly-Cys-Hyp-D-Trp-Glu-Pro-Trp-Cys-NH$_2$

SEQUENCE 1

where Hyp = 4-*trans*-hydroxyproline.

The presence of D-tryptophan in position 4 of contryphan was confirmed by chemical synthesis. The post-translational epimerization in all other D-amino acid-containing small peptides characterized previously from vertebrates and molluscan systems is in position 2.

The standard amino acids in polypeptides translated from genes are exclusively in the L-configuration. In recent years it has been established that D-amino acids can be post-translationally introduced into such polypeptides (1). Several small peptides have been characterized, which contain a D-amino acid. The first of these was dermorphin, a potent heptapeptide agonist of the μ-opiate receptor from amphibian skin, discovered by Erspamer and co-workers (2). A number of other peptides from amphibian skin (including the deltorphins and bombinin-H) were also found to have a D-amino acid. The cDNAs encoding these peptides were characterized (3, 4). The results demonstrated unequivocally the presence of mRNA encoding the peptide precursor, indicating that the D-amino acid was post-translationally formed from the corresponding L-isomer.

In addition to these vertebrate systems, small peptides with D-amino acids have also been described in invertebrate systems, primarily molluscs. An FMRFamide analog from the bivalve, *Mytilus edulis*, which contains a D-leucine, has been characterized (5). Likewise, the land snail *Achatina fulica* has D-amino acid-containing small peptides, achatin-I and fulicin (6, 7). The cDNA encoding the precursor of fulicin was found to contain the usual L-Asn codon at the D-Asn position (8). Recently, the post-translational inversion of an amino acid was demonstrated *in vitro* for ω-agatoxin-IVB (also termed ω-agatoxin-TK), a calcium channel inhibitor from funnel web spider (9). The peptide isomerase that preferentially acts on Ser^{46} of the 48-amino acid peptide has been isolated and characterized.

The small peptides which appear to be post-translationally modified to convert an L- to a D-amino acid from a variety of phylogenetic systems are shown in Table I. Although there is no homology between vertebrate and invertebrate peptides (and the three molluscan peptides exhibit no sequence similarity), in every case the D-amino acid is found in the second position. This suggests that for small D-amino acid-containing peptides, the proteolytic event that generates the mature peptide and the post-translational enzymatic system that converts an L- to a D-amino acid work in combination to always generate the D-amino acid at position 2.

In this report, we describe the purification and characterization of a novel D-amino acid-containing peptide from the venom of *Conus radiatus*, which causes a "stiff-tail syndrome" in mice. This octapeptide, contryphan, has a D-tryptophan residue. This is the first report of D-tryptophan being formed through post-translational modification. Furthermore, in contrast to all of the small D-amino acid-containing peptides shown in Table I, contryphan does not have the D-amino acid in position 2. Like most peptides found in *Conus* venoms, contryphan is cross-linked by a disulfide bond; all other peptides in Table I are not disulfide-crosslinked. The discovery and characterization of contryphan indicates that the modification system for converting L- to D-amino acids evolved in *Conus* venom ducts differs significantly from the post-translational isomerization of an L-to a D-amino acid described previously for all other small peptides in both vertebrate and previously characterized molluscan systems.

MATERIALS AND METHODS

Preparation of Venom Extract—Specimens of *C. radiatus* were obtained from the Philippines. The venom ducts were dissected from the cone snails, and the venom was squeezed out of the ducts as described previously (10). The collected venom was lyophilized and stored in the freezer. Five-hundred milligrams of lyophilized venom was sequentially extracted with 10 ml each of water, 20% acetonitrile, 40% acetonitrile, and 60% acetonitrile. The venom suspension was sonicated in the extracting solvent for three 30-s periods over ice water and centrifuged at 5000 × *g* for 5 min. The combined supernatants were stored at −20 °C for further purification.

Purification of Peptides—Crude venom extract was applied onto a Vydac C$_{18}$ semi-preparative column (10 × 250 mm) and eluted with a linear gradient of acetonitrile in 0.085% trifluoroacetic acid at 5 ml/min. Further purifications of bioactive peaks were done on a Vydac C$_{18}$ analytical column (4.6 × 250 mm) eluted with the acetonitrile/trifluoroacetic acid system at 1 ml/min. The effluents were monitored at 220 nm. Peaks were collected in polypropylene tubes and aliquots were assayed for biological activity.

Bioassay—Biological activity was assayed by intracranial injection in mice (9–21 days old). Aliquots (20–30 μl) of peptide in normal saline were injected using a 0.3 ml syringe with a 29-gauge needle. Each control mouse was injected with an equal volume of normal saline solution containing dissolved residue of lyophilized column buffer. After injection, the mice were placed in cages for observation.

Peptide Sequencing—The peptide was reduced and alkylated prior to sequencing. The pH of the peptide solution was raised to 7–8 by adding 0.5 M Tris base before dithiothreitol was added to a final concentration of 10 mM. The solution was flushed with nitrogen, incubated at 65 °C for 20 min, and cooled to room temperature. Three microliters of 4-vinylpyridine were added per 500 ml of solution. The mixture was left in

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TABLE I
Primary structure of small peptides containing D-amino acids

The underlined letters represent D-amino acid residues; * denotes COOH-terminal amide. All the rest are standard single letter codes for amino acid residues. *P.* = *Phyllomedusa*; *A.* = *Achatina*; *M.* = *Mytilus*.

Peptide	Sequence	Source	Phylum	Ref.
Dermorphin	Y$\underline{A}$FGYPS*	*P. sauvagei*	Vertebrata	2
Met-deltorphin	Y$\underline{M}$FHLMD*	*P. sauvagei*	Vertebrata	20, 21
Achatin-I	G$\underline{F}$AD	*A. fulica*	Mollusca	6
Fulicin	F$\underline{N}$EFV*	*A. fulica*	Mollusca	7
Mytilus-FFRFamide	A$\underline{L}$AGDHFFRF*	*M. edulis*	Mollusca	5

the dark for 25 min at room temperature then diluted with 0.1% trifluoroacetic acid and applied onto a Vydac C_{18} analytical HPLC[1] column.

The purified peptide was sequenced by automated Edman degradation (11) on an Applied Biosystems 477A protein sequencer with a 120A analyzer (DNA/Peptide Facility, University of Utah). The 3-phenyl-2-thiohydantoin derivatives were identified by HPLC. The predicted mass of the peptide was compared with the molecular masses obtained by Dr. Anthony Craig of the Salk Institute for Biological Studies using laser desorption mass spectrometry and liquid secondary ionization mass spectrometry (LSIMS). The LSIMS samples were measured at 3000 resolution from a glycerol matrix.

Peptide Synthesis—The protected peptide resin was synthesized using standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry and couplings using equimolar amounts of amino acid derivatives dicyclohexylcarbodiimide and hydroxybenzotriazole. Some amino acid side chains were protected as follows: Cys (trityl), OH-Pro (*t*-butyl), and Glu (*t*-butyl).

After synthesis, the terminal Fmoc group was removed by treatment with 1:4 piperidine:*N*-methylpyrrolidone (v/v). The peptide was cleaved from the resin by treatment with trifluoroacetic acid/H_20/ethanedithiol/phenol/thioanisole (80/5/2.5/7.5/5 by volume) for 1.5 h at room temperature. The mixture was filtered under vacuum into *t*-butyl methyl ether at −10 °C. Linear peptide was collected by centrifugation at 5000 × *g* for 5 min and washed with *t*-butylmethyl ether. The pellet was dissolved in 20% acetonitrile containing 0.1% trifluoroacetic acid and applied into a Vydac C_{18} preparative column (25 × 250 mm). Elution was carried out at 20 ml/min using a gradient of acetonitrile in 0.1% trifluoroacetic acid. The major peptide-containing fraction was oxidized with 1 mM iodine in 0.1% trifluoroacetic acid, 20% acetonitrile (12), then the reaction was quenched with a few drops of 0.1 M ascorbic acid. The oxidized peptide was purified by HPLC.

RESULTS

Purification of Contryphans—Crude venom was obtained from specimens of *C. radiatus* collected from Manila Bay and Marinduque. The crude venom extract prepared as described under "Materials and Methods" was applied to a C_{18} semi-preparative column, using a gradient of 0.45% CH_3CN/min. A complicated HPLC profile, typical of *Conus* venoms was obtained (see Fig. 1*A*). When injected intracranially in mice, material from the peaks indicated by the *arrows* caused mild excitatory symptoms at low doses with tail raising and severe symptoms including "barrel rolling" and seizure observed with increasing dosages (see Table II).

The peak designated as *R* was resolved into several peaks on HPLC in a C_{18} analytical column eluted with a very slow gradient of acetonitrile. The first major peak, which showed the same activity as the *R peak* of *A* was rechromatographed to give a homogeneous peak shown in *C*. Similar HPLC runs of the *R1 peak* of *A* gave the profile in *D*; rechromatography of this peak gave a homogeneous peptide.

Biochemical Characterization and Biological Activity of Contryphan and Its Des-[Gly[1]] Derivative—The purified peptides were reduced, alkylated, and sequenced as described under "Materials and Methods." The amino acid sequence obtained for peak R is shown in Table III. Because of the high tryptophan content of this peptide, we designate it as "contryphan." Peptide R1 had an identical sequence except that it lacks the NH_2-terminal Gly residue. Thus, the amino acid sequences of the two peptides are as follows.

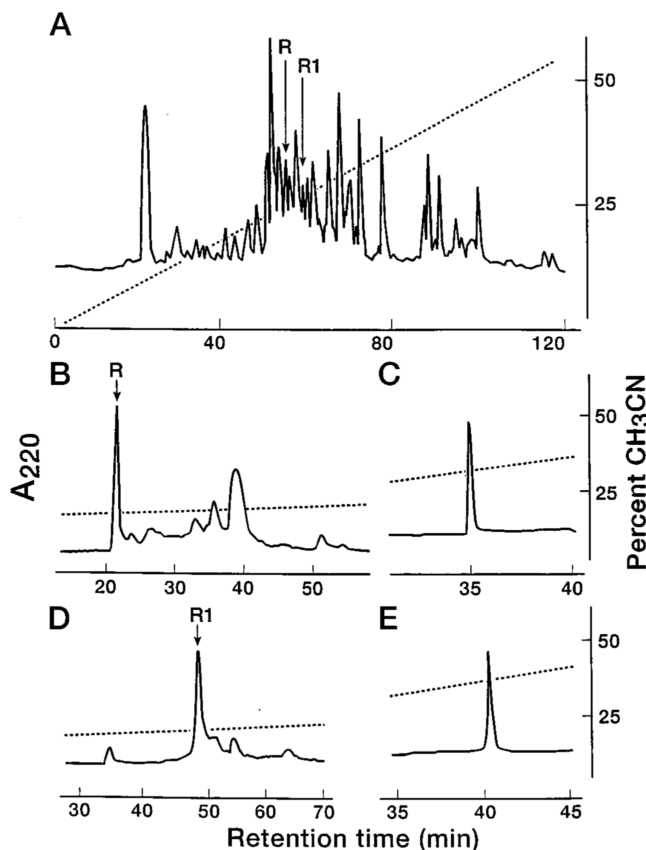

FIG. 1. **Purification of contryphan.** *A*, HPLC chromatogram of *C. radiatus* venom sequentially extracted with 10 ml each of H_20 and 20%, 40%, and 60% ACN. Five milliliters of the combined extract from 500 mg of lyophilized venom was applied onto a C_{18} semi-preparative column and eluted at 5 ml/min using a linear gradient of ACN in 0.085% trifluoroacetic acid (0.45% rise in ACN/min). The peaks corresponding to contryphan (*R*) and des-[Gly[1]]contryphan (*R1*) are indicated by *arrows*. *B*, HPLC chromatogram showing the purification of contryphan. The bioactive fraction obtained from HPLC of crude venom extract was applied onto a C_{18} analytical column and eluted at 1 ml/min using a linear gradient of ACN in 0.085% trifluoroacetic acid (0.09% rise in ACN/min). *C*, the peak was repurified at 1 ml/min with a gradient of 0.9% rise in ACN/min. *D*, HPLC chromatogram showing the purification of des-[Gly[1]]contryphan. The bioactive fraction obtained from an HPLC of crude venom extract was applied into C_{18} analytical column at 1 ml/min using a linear gradient of ACN in 0.085% trifluoroacetic acid (0.09% rise in ACN/min). *E*, the peak indicated by the *arrow* in *D* was repurified at 1 ml/min at a gradient of 0.9% rise in ACN/min.

Contryphan	GCOWEPWC-NH_2
Des-[Gly[1]]contryphan	COWEPWC-NH_2

Liquid secondary ionization mass spectrometry of the native contryphan peptide gave a monoisotopic MH^+ of 990.3 (calcu-

[1] The abbreviations used are: HPLC, high performance liquid chromatography; ACN, acetonitrile; LSIMS, liquid secondary ionization mass spectrometry.

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TABLE II
Bioactivity of contryphan

The dose ranges of contryphan shown were injected into mice as described under "Materials and Methods."

Dose	Activity
Low: 0.5–2.5 nmol/g	Grooming, licking and biting of paws, occasional tail-raising, occasional hyperactivity.
Medium: 4–6 nmol/g	Stiffening and raising of tail to almost perpendicular to body; barrel-rolling, and subsequent passivity.
High: 8–20 nmol/g	Paralysis of extremities, circular motion, barrel-rolling, seizure, and death.

TABLE III
Sequence analysis of contryphan

Sequence analysis and mass spectrometry were carried out as described under "Materials and Methods." Hyp = 4-*trans*-hydroxyproline. The calculated MH^+ value assumes that the Cys residues form a disulfide and an amidated COOH-terminal residue.

Cycle	Peak R	
	Assigned residue	Yield
		pmol
1	Gly	83.6
2	Cys	106.0
3	Hyp	91.3
4	Trp	7.7
5	Glu	23.8
6	Pro	13.2
7	Trp	1.6
8	Cys	4.9
MH^+ observed: 990.3; MH^+ calculated: 990.4		

lated MH^+ = 990.4) and a monoisotopic MH^+ of 933.3 (calculated MH^+ = 933.3) for des-[Gly[1]]contryphan. These values are consistent with the amino acid sequences assigned with the Cys residues present as disulfides and the COOH termini as amide groups.

To independently verify the sequence, synthetic peptides containing standard L-amino acids were prepared as described under "Materials and Methods." However, the synthetic peptides did not co-elute with native contryphan. The all-L analog of contryphan showed an unusually broad HPLC peak, and it appeared to be less stable than the native peptide; flushing the synthetic peptide solution with nitrogen seemed to retard its degradation to a purple derivative. Because of the identical mass of the native and synthetic L-amino acid-containing peptide, synthetic peptide homologs containing D-tryptophan at positions 4 and 7 of contryphan were synthesized. Both the fully reduced and the oxidized/folded forms of the synthetic D-Trp[4] peptide co-eluted with the corresponding forms of the natural peptide (see Fig. 2). The D-Trp[4,7] and D-Trp[7] analogs did not co-elute with native contryphan (Fig. 3). The presence of D-tryptophan in position 3 of des-[Gly[1]]contryphan was also confirmed by chemical synthesis and co-elution of native and synthetic material.

On intracranial injection into mice, synthetic contryphan elicited identical symptomatology to the native material (tail raising and hyperactivity) at low doses. Surprisingly, the all-L analog, [L-Trp[4]]contryphan shows a similar activity, whereas [D-Trp[7]]contryphan elicited no detectable activity (>10 nmol/g body weight). Des-[Gly[1]]contryphan elicited similar symptoms to contryphan but appears to be less potent; even at the highest doses (20 nmol/g), the peptide did not cause any lethality.

DISCUSSION

The data presented above document the purification, characterization, and chemical synthesis of two homologous peptides, contryphan and des-[Gly[1]]contryphan from the venom of *Conus radiatus*. The most striking feature of these peptides is

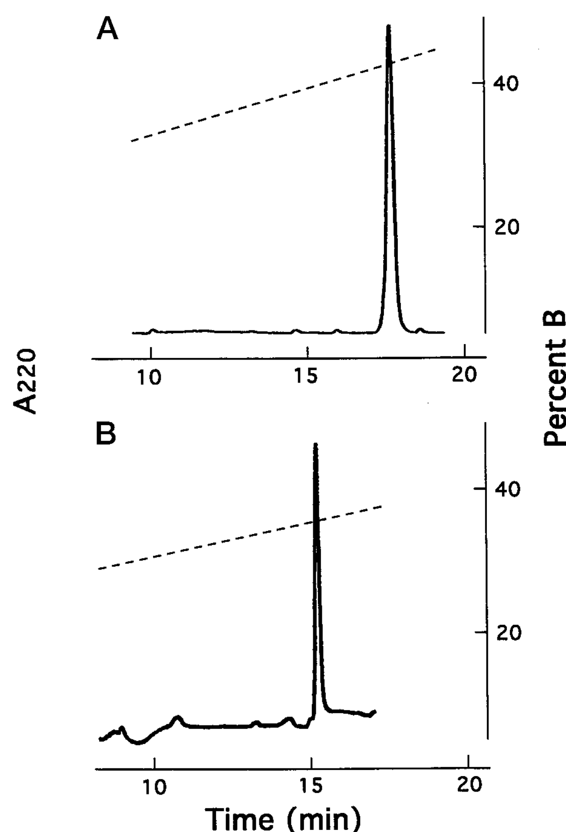

FIG. 2. **HPLC co-elution of synthetic and naturally occurring contryphan.** Gradients are represented by *dotted lines* showing the rise in percent of buffer B (90% ACN, 0.085% trifluoroacetic acid). *A*, linear forms of synthetic peptide and contryphan reduced with dithiothreitol (1.25% increase in buffer B/min). *B*, oxidized form of synthetic peptide with native contryphan (1% increase in buffer B/min).

the presence of D-tryptophan, the first occurrence of this unusual amino acid in a *Conus* peptide. Indeed, as far as we are aware, D-tryptophan has not been described previously in any mRNA-encoded polypeptidic structure. Analysis of a cDNA clone from a *C. radiatus* venom duct library encoding contryphan has shown that D-Trp is coded for by the standard codon for L-Trp.[2] Previous reports of the occurrence of D-tryptophan are in peptide-like structures, which are almost certainly not translated through the normal ribosomal route, such as in the fungal aselacins, which are cyclic pentapeptolides (13).

In recent years it has been demonstrated that D-amino acids can be introduced into small peptides through post-translational modification (1). The sequences of D-amino acid-containing small peptides is shown in Table I. Contryphan is distinct from D-amino acid-containing peptides previously characterized in several respects. It appears to be the only small peptide (≤10 amino acids) containing a D-amino acid residue, which is also disulfide-crosslinked. Furthermore, in other small peptides, although a variety of different D-amino acids have been found (including alanine, asparagine, methionine, phenylalanine, and leucine), the D-amino acid is always the penultimate NH_2-terminal amino acid (Table I). It has been suggested that the isomerase for the Leu → Asp amino acid conversion may cue into the dibasic signal for proteolytic processing of peptide precursors (1), post-translationally converting the second amino acid into the D-configuration regardless of the identity of the amino acid at the designated locus. For contryphan the number 2 position rule could not apply, since the D-amino acid

[2] M. Watkins, E. C. Jimenéz, and D. R. Hillyard, unpublished results.

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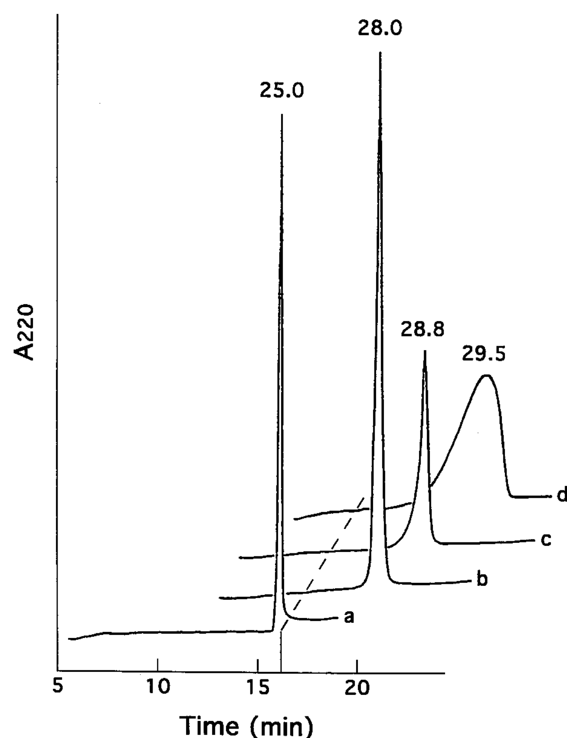

FIG. 3. **Reverse-phase HPLC chromatograms of contryphan and analogs.** *a*, contryphan; *b*, [D-Trp[7]]contryphan; *c*, [L-Trp[4],D-Trp[7]]contryphan; *d*, [L-Trp[4]]contryphan. Peptides were applied to a C_{18} analytical column (Vydac, 218TP54, 4.6 x 250 mm) and eluted using a linear gradient from 13.5 to 40.5% ACN in 0.08% trifluoroacetic acid. Peaks are labeled with the apparent percent ACN at which elution occurred, calculated from the elution time. The elution position of contryphan relative to its analogs is indicated by the *dotted line*. Mixing experiments (not shown) demonstrate that authentic contryphan is well resolved from the all-L, D-Trp[7], or L-Trp[4],D-Trp[7] homologs, but the three homologs are not well resolved from each other under these elution conditions.

is Trp[4].

Thus, although *C. radiatus* is a mollusc like *Achatina* or *Mytilus*, the modification system in *C. radiatus* venom ducts for the isomerization of Trp[4] in contryphan must use a different positional cue. It is relevant to note that in two arthropod systems, very much larger polypeptides with D-amino acids have been described, including a lobster neurohormone (14), as well as the spider toxin ω-agatoxin IVB (15, 16). In both of these systems, the D-amino acid is not found in position 2. For ω-agatoxin IVB, which comprises 48 amino acids, the conversion of Ser[46] to the D-isomer has been found to enhance toxin stability as well as potency as a calcium channel blocker (15, 17). In this case, an isomerase shown to be homologous to serine proteases has been purified from the spider venom ducts, which carries out the Leu → Asp conversion (9, 18). It will be of interest to compare the contryphan modification system with the spider enzyme and the enzymes carrying out the fixed positional modification found in the small molluscan and frog skin peptides. In contryphan, pairs of proline and tryptophan residues are present, but only the more NH_2-terminal amino acid residues are post-translationally modified (proline to hydroxyproline and L-tryptophan to D-tryptophan). The more COOH-terminal residue remains unmodified in each case. These results suggest that such modification enzymes distinguish between closely apposed residues.

The discovery of contryphan raises the question of whether other peptides with amino acids that have been post-translationally modified to the D-configuration exist in *Conus* venoms. We should note that since no homolog with L-tryptophan was found in *C. radiatus* venom, indicating that it is either absent or present at much lower levels than the D-Trp isomer. Preliminary results suggest that contryphans are distributed more widely in *Conus* venoms, homologous peptides have been found in venom ducts collected from other *Conus* species.[3] Thus, the ability to post-translationally modify an L- to a D-amino acid may be widespread in *Conus*, and additional D-amino acid-containing peptides unrelated to contryphan could well be present in *Conus* venoms.

When injected into the mouse central nervous system, contryphan elicits characteristic symptoms such as the stiff-tail syndrome (Table II). At higher doses, more generalized excitatory effects (*i.e.* barrel rolling and seizures) are induced. The mechanistic basis for the symptomatology observed is presently unknown. Recently, we suggested that at least some of the excitatory activities present in the venoms of fish-hunting cone snails may act together to cause an excitotoxic shock response in the prey (19), which leads to very rapid immobilization of the fish prey. Given the excitatory effects of contryphan in the mammalian central nervous system, a possible function for this peptide in the venom is to contribute to the excitotoxic shock response.

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