Research Journal of Biological Sciences 2 (4): 462-467, 2007

The Optimization of Ultrasonic Wave Extraction and Vacuum Liquid Chromatography for Isolation of Destruxins

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Abstract: Destruxins, a family of cyclic peptide mycotoxins, have been being paid more and more attention for their multiple bioactivities such as insecticide, antivirus and immunomodulation. In order to decrease the consumption of solvents and time to isolate and purify destruxins A and B (DA and DB), Ultrasonic Wave Extraction (UWV) and Vacuum Liquid Chromatography (VLC) were employed. Under the optimal conditions of pH 4-5, solvent (dichloromethane/ethyl acetate, v v^{-1} = 1/1)/broth (v v^{-1}) between 1-1.5 and extracted time in 45-90 min, UWV gave an excellent recovery (extracting efficiency > 90%). Meanwhile, in the VCL experiment, the gradient of hexane/acetone 100 /0-93/7 as eluant could isolate DA and DB clearly. However, the eluants of dichloromethane/methanol and hexane/ethyl acetate were not as good as hexane/acetone.

Key words: Destruxins, Ultrasonic Wave Extraction (UWV), Vacuum Liquid Chromatography (VLC), isolation of destruxins

INTRODUCTION

Destruxins, a family of cyclic peptide toxins, which may play an important role in pathogenesis of *Metarhizium anisopliae* and *Alternaria brassicae* (Pedras *et al*., 2000; Milner *et al*., 2002). These compounds are typically composed of five amino acids and a á-hydroxy acid forming a cyclic hexadepsipeptide. The general formula of destruxins is cyclo (-D-HA-L-Pro-L-Ile-L-MeVal-L-MeAla-β-Ala-), where HA represents a D-á-hydroxy acid residue. To date, 36 destruxins have been reported (Pedras *et al*., 2002; Vazquez *et al*., 2005). They were found from different fungi, but the most extensively reported fungus was *M. anisopliae*. Some destruxins, especially destruxin A, E and B (DA, DE, DB) showed insecticidal activities (Thomsen and Eilenberg, 2000; Hu *et al*., 2007). DB and desmethyl-DB were phytotoxic to the plants of *Brassica* (Pedras *et al*., 2000). DB also had suppressive effects on hepatitis B virus surface antigene gene expression in human hepatoma cells (Chen *et al*., 1997). In addition, destruxins showed erythropoietin-inducing and immunomodulating activities (Cai *et al*., 1998) and anti-resorptive effect for osteoclasts (Yoshimoto and Imoto, 2002).

Destruxins have been being paid enough attention for the multiple bioactivities. However, the isolation and purification of destruxins still fall into the complicated processes, which consume a lot of toxic solvents and times but have low recovery. In this experiment, we aimed to optimize the conditions of Ultrasonic Wave (UW) extraction and Vacuum Liquid Chromatography (VLC) so as to improve the preparing process of destruxins.

MATERIALS AND METHODS

Ultrasonic wave extraction

Broth used: The *M. anisopliae* strain MaQ10 was cultured with the previous methods (Hu *et al*., 2006). After filtered with a vacuum filter and removed the pellets, the broth was stand-by. Meanwhile, the concentrations of destruxins A and B in the broth were quantified, by means of HPLC, as 251.5 and 49.8 mg L^{-1}, respectively.

Optimization of extraction conditions: The Response Surface Methodology (RSM) was used to optimize of extraction conditions of destruxins. Based on the predecessors experiences (Pais *et al*., 1981; Loutelier *et al*., 1996) three factors, the value of pH, rate of solvent/broth [v v^{-1}, the solvent is the mixture of dichloromethane and ethyl acetate (v v^{-1} = 1/1)] and the time to extract under UW 40 kHz, were selected as independent variables in the Central Composite Design (CCD) experiment. Each factor had five coded

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levels (-1.6818,-1, 0, 1, 1.6818). The CCD contained a total of 20 treatments that included 8 factorial, 6 axial and 6 central points for replication (Table 1). The value of broth in each treatment was 100 mL. The response was the extraction efficiency of DA or DB, which was evaluated according to the follow formula:

Extraction efficiency of DA or DB (%) = $100 \times M_O \div M_F$

Where M_O was the quantity of DA or DB in organic phase after extracted, while M_F was the quantity of DA or DB in broth before extracted.

DA and DB were quantified with HPLC refer to Hu *et al.* (2006). The response value (Y) of each trial was the average of duplicates. The statistical software DPS (Data Processing System, Version 3.01) (Tang and Feng, 2002) was used to analyze the experimental data. After the optimal extraction conditions were evaluated, a new trail was carried out to test the result actuality.

Vacuum Liquid Chromatography (VLC)

Sample preparation: A kind of Crude Destruxins (CD) was used to experiment. Each sample had 1 g CD. There were 135 mg DA (13.5 %) and 26 mg DB (2.6 %) in 1 g CD. To prepare sample, 1 g CD was diluted in dichloromethane, mixed with 4 g silica gel (100) and dried for stand-by.

Device of VLC: A flask connected a column and vacuum pump made of a device for VLC (Fig. 1).

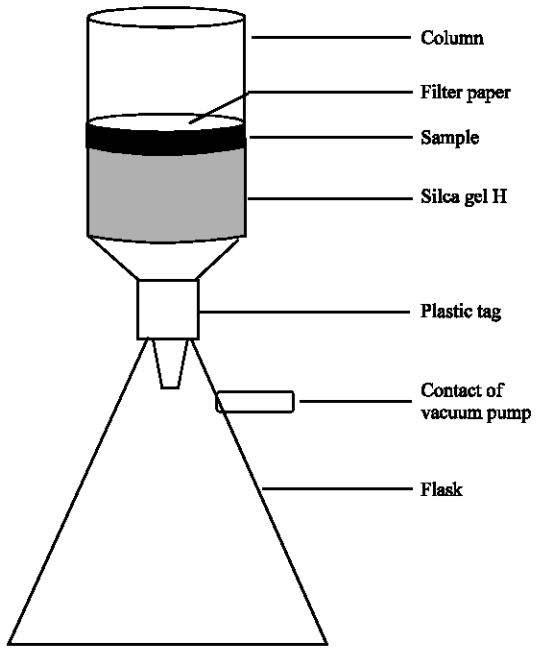

Fig. 1: Device of VLC

Column fill: A column of 20 mm diameter was filled with dry silica gel H 40 g. After the silica gel was pressed to close together, a sample was added on the top, then a piece of filter paper was covered on the surface of sample to avoid being moved when pouring solvent.

Elution: Three kinds of eluent, dichloromethane/ methanol = 100/0-93/7, hexane/acetone = 100/0-30/70 and hexane/ethyl acetate = 100/0-30/70, were selected as flow phases. Each fraction was collected 100 mL and DA and DB were quantified by means of HPLC (Hu *et al.*, 2006).

The same experiment was replicated twice, the mean of the two trails was carried out to analyze. The flowing-curves were employed to describe the difference from three flow phases, in which flow phase was taken as x-axis and concentration of DA or DB as y-axis.

RESULTS AND DISCUSSION

Optimization of ultrasonic wave extraction

Destruxin A: The extraction efficiency had very great difference in different treatments (Table 1). A regressive Eq. 1 indicated the response (y) and pH (X_1), volume rate of solvent/broth and extracted times, was given out by DPS software.

$$Y = 92.58 \ 5.74X_1 + 5.15X_2 + 5.79X_3 \ 9.40X_1^2 \ 4.30X_2^2 \ 4.02X_3^2 \ 1.28X_1X_2 \ 1.37X_1X_3 \ 0.53X_2X_3 \quad (1)$$

That equation could be accepted after subjected Analysis of Variance (ANOVA) (Table 2). The F1 from lack-in-fit was 54.991 which showed a probability $p<0.01$ to indicate a good fit. Meanwhile, F2 indicated the model was a best regressed because the value of F2 was 4.742 and the $p<0.01$. However, the term X_2X_3 was insignificant (p-0.36>0.05) and should be removed from the Eq. 1. So, the Eq. 1 could simplified as Eq. 2:

$$Y = 92.58 - 5.74X_1 + 5.15X_2 + 5.79X_3 - 9.40X_1^2 \ -4.30X_2^2 - 4.02X_3^2 - 1.28X_1X_2 - 1.37X_1X_3 \quad (2)$$

According to the interaction between pH, solvent/ broth and extracted time (Fig. 2), the optimal extraction conditions could be evaluated. When the extraction efficiency > 90%, the Ph and solvent/broth must be 3.0-5.0 and 0.8-1.84, while the extracted time fall into 40-110.45 min. However, it was not necessary to raise the extraction efficiency by means of increasing consumption of solvent and extracted time. So, Ph-4.0 (code was-0.5), solvent/ broth = 1 (code was 0) and extracted time = 60 min (code

Table 1: The 3-factor central composite design and responses for optimization of extraction

Treatment.	Coded values			Actual values			Reponses (%)	
No.	X_1	X_2	X_3	pH	Solvent/broth	Time (min)	DA	DB
1	1.0000	1.0000	1.0000	7.00	1.50	90.00	71.5	76.1
2	1.0000	1.0000	-1.0000	7.00	1.50	30.00	68.8	71.5
3	1.0000	-1.0000	1.0000	7.00	0.50	90.00	71.2	74.6
4	1.0000	-1.0000	-1.0000	7.00	0.50	30.00	66.1	68.1
5	-1.0000	1.0000	1.0000	3.00	1.50	90.00	94.1	99.2
6	-1.0000	1.0000	-1.0000	3.00	1.50	30.00	85.6	87.6
7	-1.0000	-1.0000	1.0000	3.00	0.50	90.00	88.4	92.5
8	-1.0000	-1.0000	-1.0000	3.00	0.50	30.00	78.1	79.9
9	-1.6818	0.0000	0.0000	1.64	1.00	60.00	64.5	64.1
10	1.6818	0.0000	0.0000	8.36	1.00	60.00	58.7	61.5
11	0.0000	-1.6818	0.0000	5.00	0.16	60.00	59.9	57.1
12	0.0000	1.6818	0.0000	5.00	1.84	60.00	92.1	90.2
13	0.0000	0.0000	-1.6818	5.00	1.00	9.55	61.2	57.8
14	0.0000	0.0000	1.6818	5.00	1.00	110.45	92.4	94.2
15	0.0000	0.0000	0.0000	5.00	1.00	60.00	93.4	97.3
16	0.0000	0.0000	0.0000	5.00	1.00	60.00	94.2	96.8
17	0.0000	0.0000	0.0000	5.00	1.00	60.00	91.8	98.2
18	0.0000	0.0000	0.0000	5.00	1.00	60.00	92.5	97.8
19	0.0000	0.0000	0.0000	5.00	1.00	60.00	94.6	99.1
20	0.0000	0.0000	0.0000	5.00	1.00	60.00	90.5	96.4

Table 2: Analysis of Variance (ANOVA) for response surface quadratic model to optimize extraction

Source of	DA					DB				
variance	SS	DF	MS	F	p	SS	DF	MS	F	p
X_1	12585.42	1.00	12585.42	188.36	0.00	40254.62	1.00	40254.62	407.81	0.00
X_2	10146.47	1.00	10146.47	151.86	0.00	42138.21	1.00	42138.21	426.89	0.00
X_3	12816.98	1.00	12816.98	191.82	0.00	69846.04	1.00	69846.04	707.59	0.00
X_1^2	35616.81	1.00	35616.81	533.05	0.00	140922.47	1.00	140922.47	1427.65	0.00
X_2^2	7475.69	1.00	7475.69	111.88	0.00	52000.95	1.00	52000.95	526.81	0.00
X_3^2	6525.54	1.00	6525.54	97.66	0.00	38463.31	1.00	38463.31	389.66	0.00
X_1X_2	364.08	1.00	364.08	5.45	0.04	1155.15	1.00	1155.15	11.70	0.01
X_1X_3	423.44	1.00	423.44	6.34	0.03	2196.52	1.00	2196.52	22.25	0.00
X_2X_3	61.73	1.00	61.73	0.92	0.36	107.64	1.00	107.64	1.09	0.32
Model	2851.57	9.00	316.84	F2 = 4.742	0.01	3455.16	9.00	383.91	F2 = 3.889	0.03
Rests	668.16	10.00	66.82			987.10	10.00	98.71		
Lack-of-fit	656.23	5.00	131.25	F1 = 54.991	0.00	982.28	5.00	196.46	F1 = 203.792	0.00
Errors	11.93	5.00	2.39			4.82	5.00	0.96		
Total	3519.73	19.00				4442.26	19.00			

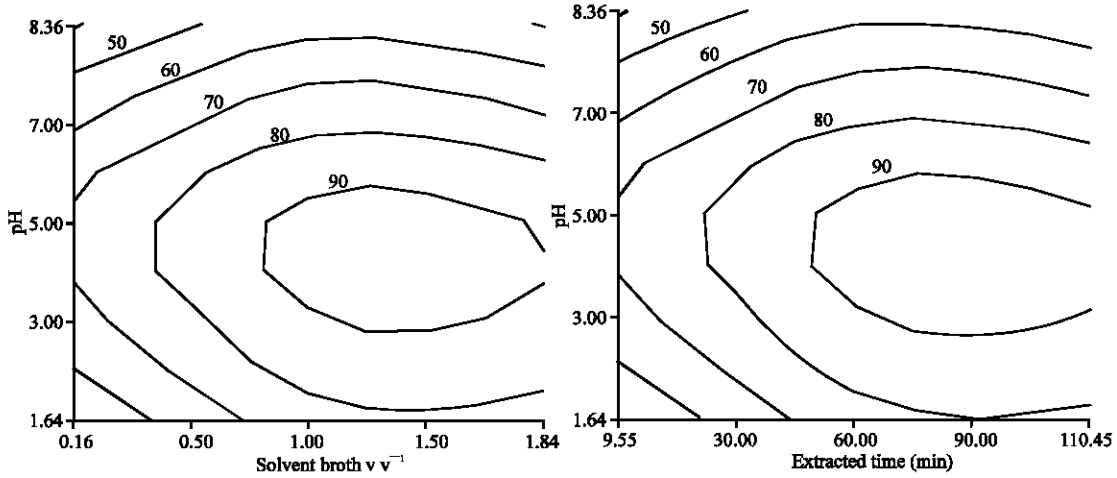

Fig. 2: Effects of interaction between pH, solvent/broth and time on the extraction efficiency of destruxin A

Table 3: Results of experiments to verify the models

	Ph Solvent/broth extracted time						Predict value		Observation value	
Treatments	Code	Actual	Code	Actual	Code	Actual	DA	DB	DA	DB
A	-0.5	4	0.5	1.25	-0.5	45	90.68	93.79	90.18	93.56
B	-0.5	4	0	1	0	60	93.10	97.45	93.58	96.94
C	0	5	1	1.50	1	90	97.04	100	96.14	98.21
D	0	5	0	1	1	90	95.56	100	95.45	98.33

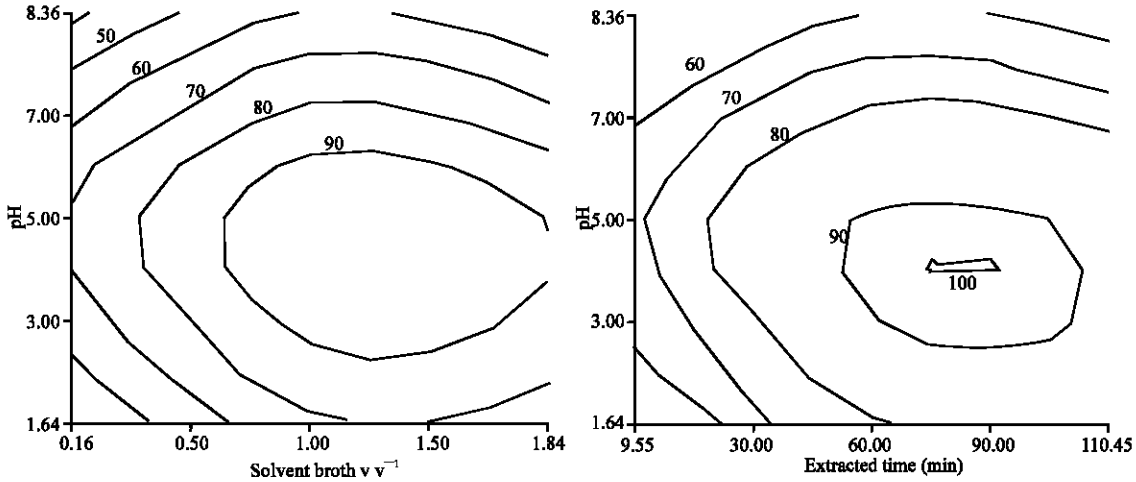

Fig. 3: Effects of interaction between Ph, solvent/broth and time on the extraction efficiency of destruxin B

was 0) were selected, under the condition the predict extraction efficiency was 93.10% and actual value was 93.58% (Table 3).

Destruxin B: A quadratic regressive curve (3) was given out to model the relationship between the extracting efficiency (Y) and Ph (X_1), solvent/broth (X_2) and time (X_3) (Fig. 3):

$$Y = 97.21 - 5.37X_1 + 5.49X_2 + 7.07X_3 - 9.77X_1^2 - 5.94X_2^2 - 5.11X_3^2 - 1.19X_1X_2 - 1.64X_1X_3 - 0.36X_2X_3 \quad (3)$$

That Analysis of Variance (ANOVA) (Table 2) indicated that the model could be accepted but the term X_2X_3 should be deleted for its insignificant (p-0.36 > 0.05). Therefore, a new simple Eq. 4 was:

$$Y = 97.21 - 5.37X_1 + 5.49X_2 + 7.07X_3 - 9.77X_1^2 - 5.94X_2^2 - 5.11X_3^2 - 1.19X_1X_2 - 1.64X_1X_3 \quad (4)$$

Similar with destruxin B, according to the interaction between Ph, solvent/broth and extracted time, (Fig. 1), the optimal extraction conditions were Ph-4.0 (code-0.5), solvent/broth = 1 (code 0) and extracted time = 60 min (code 0), the predict extraction efficiency was 97.45% and the actual observation value was 96.94% (Table 3).

Vacuum liquid chromatography: There were apparent different flow curves under different elution (Fig. 3). In the gradient of dichloromethane/methanol (Fig. 4-A), DB could be detected at 100/0-96/4 with a total recovery 94.73%, but DB was mainly collected at 99/1 with 87.19% recovery (Fig. 4-D), however, little of DA was mixed there (31.60 mg L^{-1}). DA appeared in 100/0-93/7 at a 95.12% recovery and was principally collected at 97/3 with a 60.64% recovery.

Under the elution of the gradient of hexane/acetone (Fig. 4-B), DB was detected at 100/0-70/30 with a total recovery 96.17% and mainly collected at the fraction of 90/10 with 68.50% recovery (Fig. 4-D). DA appeared in the fractions of 80/20-30/70 with a 96.76 % recovery and was principally collected at 70/30-50/50 with a 88.54 recovery.

Different from the formers, in the gradient of hexane/ethyl acetate (Fig. 4-C), DB recovered 96.04% at 100/0-70/30 and mainly collected at 90/10 in which mixed lots of DA (56.95 mg L^{-1}). DA was mass collected in 80/20-70/30 with blend of DB.

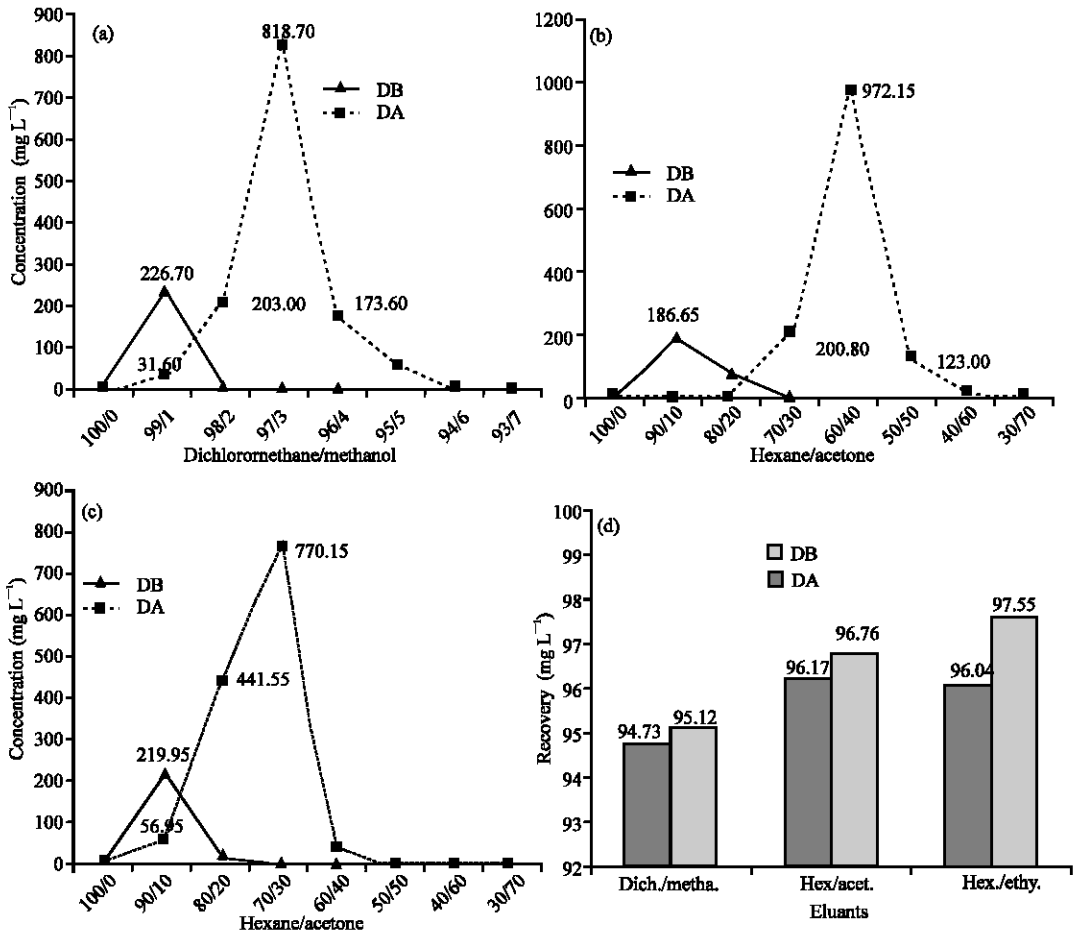

Fig. 3: The flow curves and recovery

The gradient of hexane/acetone could be determined as the best elution, because hexane/ethyl acetate and dichloromethane/methanol could not isolate DA and DB very well, although they had larger recovery and less consumption of solvents.

DISCUSSION

To extract destruxins from broth, researchers employed various solvents. Dichloromethane was often used (Pais *et al*., 1981; Chen *et al*., 1999) however, there were different solubility exponent among destruxins for their different polarities. Therefore, in order to increase recoveries of all destruxins, it is necessary to enlarge the consumption of dichloromethane. Consequently, Liu *et al*. (2004) selected acetonitrile as solvent because it is able to solve destruxins very well, but, acetonitrile can solve water as well, which lead to difficulty to isolate. So, it seems desirable to adopt a composite solvent. Thereby, Loutelier *et al*. (1996) used dichloromethane/ethyl acetate (v v^{-1} = 50/50) and obtained the recoveries > 90%, nevertheless, the process consume too much solvents and time.

So, in our experiment, we employed the solvents referred to Loutelier *et al*. (1996) and introduced ultrasonic wave extraction mean to shorten time and decrease consumption of solvent. In result, we got the purpose. Under the conditions of pH 3.0-5.0, solvent/broth between 0.8-1.6 and extracted time 50-90 min, the extracting efficiency will amount to more than 90%. However, because the concentrations of destruxins influenced on their recoveries, it is further required to illustrate if the conditions of extraction need to be relevantly adjusted.

There were different stationary and flow phases in column chromatographic process. Kodaira (1961) employed an alumina column with elution of benzene, while Pais *et al*. (1981) utilized silica gel column and eluant of hexane/acetone gradient, but Chen *et al*. (1999) discovered that ion-exchange together with silica gel column chromatography, then semi-preparative HPLC

gave better recoveries. However, the general chromatography spends more much time. In order to shorten the chromatographic duration, we carried out Vacuum Liquid Chromatography (VLC) that did not reported in destruxins isolation. The results showed that VLC could isolate detruxin A and B. Also, with the silica gel column, the hexane/acetone gradient was the best eluant. It applied some useful information for further exploration. However, because kinds and contents of destruxins change in different samples, correspondingly, chromatographic methods have to change according to specific sample.

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