PEROXIDASES FROM TULIP BULBS (*Tulipa fosteriana*, L.) OXIDIZE XENOBIOTICS *N*-NITROSODIMETHYLAMINE AND *N*-NITROSO-*N*-METHYLANILINE *in vitro*

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Received June 17, 1997
Accepted August 28, 1997

Tulip bulbs (*Tulipa fosteriana*, L.) contain peroxidases catalyzing the oxidation of the xenobiotics *N*-nitrosodimethylamine (NDMA) and *N*-nitroso-*N*-methylaniline (NMA). Three anionic (A_1, A_2, A_3) and four cationic (B, C, D, E) peroxidases were purified from this tissue, partially characterized and used for kinetic studies. Demethylation of NDMA and NMA producing formaldehyde is catalyzed by one anionic (A_1) and three cationic (C, D, E) peroxidases. The oxidation of NDMA by tulip peroxidases exhibits the Michaelis–Menten kinetics. The apparent Michaelis constant and the maximal velocity values for this substrate were determined. On the other hand, non-Michaelian kinetics for the NMA oxidation were observed with tulip peroxidases. The most abundant cationic peroxidase (peroxidase C) was used for detailed enzymatic studies. In addition to formation of formaldehyde, methylaniline, aniline, 4-aminophenol and phenol were found to be metabolites formed from NMA. Phenol was formed presumably by *N*-demethylation *via* a benzenediazonium ion, while methylaniline, aniline and 4-aminophenol were products of denitrosation of the substrate. The efficiencies of plant peroxidases to oxidize NDMA and NMA *in vitro* are compared with those of cytochromes P450 and discussed.
Key words: Plants; Exogenous chemicals; *N*-Demethylation; *N*-Nitrosamines; Enzyme kinetics; Enzymatic oxidation.

Plants are organisms primarily exposed to many xenobiotics, which are, at the present time, an integral part of the environment. The increasing use of pesticides and other chemicals in modern agriculture causes higher amounts of these compounds to enter into the plants and thus into the trophic chain.

During the phase I of xenobiotics biotransformation in an organism, the oxidative reactions lead to the formation of polar compounds, which can be further conjugated during the phase II of biotransformation. Another possibility is formation of toxic, mutagenic or carcinogenic metabolites, which are not conjugated and, due to their reactivity, bind covalently to biological macromolecules[1]. Hence, the oxidative reactions are crucial for further activity of xenobiotics and for understanding their fate in the organism.

It has not been exactly known which plant enzymes are the most important for the oxidation of xenobiotics in plants *in vivo*. Several types of oxidative enzymes such as mixed function oxidases having cytochrome P450 (P450) as the terminal oxidase, per-

oxidase, non-specific oxidases and lipooxygenases have been suggested to be implicated in the oxidation of xenobiotics in plants[1–6]. However, *in vitro* studies with isolated plant enzymes potentially important for xenobiotics metabolism as well as *in vivo* studies in plants are scarce[6,7].

The metabolism of xenobiotics by purified plant P450 reconstituted with NADPH : P450 reductase was studies only in a few cases. A cytochrome P450 isolated from avocado was found to metabolize xenobiotics (*e.g.* 4-chloro-*N*-methylaniline)[8]. Demethylation of this substrate was also measured in yeast transformed with recombinant cytochrome P450 CYP71 from avocado[9], demonstrating that CYP71 product oxidizes this xenobiotic. A recombinant plant cinnamate 4-hydroxylase CYP73 (from *Helianthus tuberosus* tubers) produced in yeast was highly specific for its natural substrate[10,11]. However, it also demethylated the natural substrate herniarin and oxygenated five xenobiotics including the herbicide chlorotoluron[10].

The aim of the complex study carried out in our laboratory is to find which plant enzymes can participate in the metabolism of xenobiotics in plants. *N*-Nitrosamines, which are exogeneous chemicals having toxic and carcinogenic effects for organisms, are used as the model xenobiotics in this study. Recently, we have shown that P450 isolated from a model plant (*Tulipa fosteriana*, L.) is active in the *in vitro* reductase-mediated oxidation of studied *N*-nitrosamines *N*-nitroso-*N*-methylaniline (NMA) and *N*-nitrosodimethylamine[12,13] (NDMA). Xenobiotics aminopyrine and an azo dye 1-phenylazo-2-naphthol (Sudan I) are oxidized by this enzyme preparation, too[13,14]. NMA and NDMA (as well as the other above mentioned xenobiotics) are also oxidized by peroxidase[15–20]. Horseradish peroxidase was used as a model enzyme[15–20]. However, the efficiencies of peroxidases present in tulip bulbs (the tissue, which was used as the source for plant P450 isolation)[12–14] in oxidation of the above mentioned xenobiotics are unknown at present. To answer this question, the present study is aimed at finding the activity of tulip bulb peroxidases with respect to oxidation of model *N*-nitrosamines (NMA, NDMA). Therefore, we have partially purified the peroxidases from tulip bulbs and used for further studies. We report here several their physical characteristics as well as their kinetic and catalytic characteristics with respect to NDMA and NMA as the substrates.

EXPERIMENTAL

Chemicals

Chemicals were from the following sources: acrylamide and *N,N,N′,N′*-tetramethylethylenediamine from Fluka, benzenediazonium hexafluorophosphate from Aldrich and horseradish peroxidase from Sigma. All chemicals were reagent grade or better. NMA and NDMA were synthesized as described previously[21].

Purification of Peroxidases from Tulip Bulbs

A slight modification of the procedure for isolation of peroxidases from horseradish roots described by Shannon and coworkers[22] was used. Briefly, the tulip bulbs (*Tulipa fosteriana*, L.) stored as described in our previous paper[12] (200 g) were sliced (1 mm thick) and homogenized in 400 ml of 0.7 M Na_2HPO_4. The homogenate was filtered and precipitated by ammonium sulfate. The fraction precipitating between 35–95% of ammonium sulfate saturation was collected and transferred to a CM-Sephadex C-25 column (1.5 × 30 cm) previously equilibrated with 5 mM sodium acetate, pH 4.4. The fraction that was not adsorbed in the column was designated according to Shannon *et al.*[22] as fraction A. The CM-Sephadex column was then eluted with a linear gradient consisting of 250 ml of 5 mM sodium acetate, pH 4.4, and 250 ml of 1 M sodium acetate, pH 5.1. Just before the elution system was exhausted, a second elution gradient consisting of 200 ml of 1 M sodium acetate, pH 5.1, and 200 ml of 2 M sodium acetate, pH 5.5, was introduced into the column. The fractions containing peroxidase activity where designated as fraction B, C, D and E (according to Shannon *et al.*[22]) and pooled separately. Fraction A was dialyzed against 5 mM Tris-HCl buffer, pH 8.4, and was transferred to a DEAE-cellulose DE 52 column (1.5 × 20 cm) previously equilibrated with the same buffer. Three anionic peroxidases (designated as A_1, A_2, A_3, ref.[22]) were eluted from the column with a linear gradient consisting of 200 ml of 5 mM Tris-HCl, pH 8.4, and 200 ml of 5 mM Tris-HCl, pH 8.4, containing 0.5 M NaCl.

Fraction C, which was used for more detailed characterization and kinetic measurements with NMA and NDMA, was then purified by repeated chromatography on CM-Sephadex until elution patterns indicated that tulip peroxidase C was free from other peroxidase fractions or other protein contaminants (monitored at 280 and 405 nm).

Analytical Methods

Peroxidase activity measurement was carried out in the incubation mixture containing in 1 ml: 100 μmol Tris-HCl, pH 8.0, 18 μmol guaiacol and 100 μmol H_2O_2. After 10 min incubation (37 °C), the absorbance at 436 nm was measured on a SPECORD M-42 spectrophotometer (Zeiss, Germany).

Polyacrylamide slab gel electrophoresis was performed in the presence of 0.1% (w/v) sodium dodecyl sulfate (SDS) with 8% (w/v) acrylamide as described by Laemmli[23]. Bovine serum albumin, catalase, ovalbumin, cytochrome c and phosphorylase were used as standards.

Protein concentrations were estimated according to Bradford[24] and Lowry *et al.*[25] with bovine serum albumin as a standard. Both methods were used paralelly.

Kinetic analyses were carried out using the non-linear least-square method described previously[26].

The assay mixture for the demethylation of NDMA and NMA contained, in 1 ml, 50 μmol potassium phosphate, pH 8.0, 200 μg tulip peroxidases, 0.05–3 μmol NMA dissolved in dimethyl sulfoxide or 0.2–4.5 μmol NDMA dissolved in distilled water. The mixtures were incubated for 10 min at 37 °C. The reaction was terminated by addition of 500 μl 20% trichloroacetic acid. The amount of formaldehyde formed was measured as described by Nash[27].

The incubation mixtures containing one of the nitrosamines studied (NMA) were applied to a C18 SepPak cartridge to quantify the products formed from NMA in the peroxidase system. The cartridge was washed with 0.1 M NaOH and the products eluted with 50% aqueous methanol. The products were separated by HPLC on a Separon SGXC18 column with a linear gradient of methanol in water (20–70% (v/v) methanol from 0 to 12 min, 70–100% methanol from 12 to 16 min. The absorbance of the eluent was monitored at 250 nm. Reaction products were identified by comparison of their retention times with those of authentic standards (aniline, methylaniline, phenol, 4-aminophenol). The amounts were calculated by peak areas and by comparison with external standards. Recoveries of products were around 80% after 10 min incubation in the presence of peroxidase without hydrogen

peroxide, except the recovery of phenol (it was around 60%). The recovery of phenol when benzenediazonium hexafluorophosphate was added to the incubation mixture containing peroxidase but without hydrogen peroxide was about 20% after 10 min incubation.

The spectra of tulip or horseradish peroxidases (0.23 mg/ml) were measured at 20 °C in 50 mM Tris-HCl, pH 8.0, on a SPECORD M-42 spectrophotometer (Zeiss, Germany). Absorption spectra were recorded between 250–800 nm. The total volume in the cuvettes was 1 ml. The reference cuvette contained the same buffer without peroxidases.

RESULTS

Purification and Partial Characterization of Peroxidases of Tulip Bulbs

We have partially purified the peroxidases from tulip bulbs. The pattern of tulip bulb peroxidases was similar to that of peroxidases from horseradish roots[22]. Three of seven tulip peroxidases fractions were anionic peroxidases (A_1, A_2, A_3) whereas four enzymes were cationic peroxidases (B, C, D, E).

Individual tulip peroxidase fractions were tested for homogeneity by a one-dimensional SDS-gel electrophoresis. Each isolated enzyme fraction was shown to be a non-homogeneous protein. Among the peroxidase fractions found in tulip bulbs, the most abundant enzyme is anionic peroxidase A_2 followed by cationic peroxidase C (Table I). The molecular weights of tulip peroxidases determined by SDS-gel electrophoresis are 41 200–43 700 (Table I). Except the peroxidase A_3, the other enzymes were active with respect to oxidation of a well-known peroxidase substrat guaiacol (Table I). Isolation and partial characterization of tulip peroxidases were carried out three times. The results of these experiments did not differ. Therefore, data shown in Table I are results obtained in one representative experiment. Peroxidase C was repurified by chromato-

TABLE I
Characteristics of peroxidases isolated from tulip bulbs

Peroxidase	Total peroxidase content mg	Specific activity units[a]/mg protein	Molecular weight
A_1	0.53	0.410	42 200
A_2	43.32	0.226	41 700
A_3	15.40	0	41 700
B	19.80	0.308	42 200
C	32.00	0.180	43 700
D	14.10	0.446	41 200
E	9.17	0.024	41 200

[a] nmol guaiacol/min.

graphy on a CM-Sephadex column (four times) and used for additional characterization.

Table II shows spectral characteristics of native tulip and horseradish peroxidases C, those after reaction with hydrogen peroxide and after reduction with dithionite. The peroxidases of both plants exhibit similar absorption maxima (Table II).

Oxidation of NDMA and NMA by Peroxidases of Tulip Bulbs

The results shown in Table III indicate that NMA and NDMA are *N*-demethylated in the presence of hydrogen peroxide by several tulip peroxidase fractions to produce formaldehyde. No demethylation activity was observed when hydrogen peroxide was omitted from the reaction mixture.

Formaldehyde formation was measured in the reaction medium, which contained peroxidase, hydrogen peroxide and various NDMA or NMA concentrations. The reactions of NDMA oxidation followed the Michaelis–Menten kinetics (Fig. 1). Of the tulip peroxidases used, the peroxidase C was the most efficient enzyme with respect to NDMA oxidation. With this enzyme, the highest value of the maximal velocity (V_{max}) and the lowest value of the apparent Michaelis constant (K_m) for the formaldehyde formation were found (Table IV).

Non-Michaelian saturation curves were observed when the initial velocity of NMA oxidation was plotted as function of NMA concentrations. At low NMA concentrations (0.3 mM), this substrate was efficiently *N*-demethylated. However, the peroxidase activity was decreased if the NMA concentration was increased (up to about 1 mM). Thereafter, the enzyme activity with respect to the NMA oxidation was gradually increased with the increasing concentration of this substrate (Fig. 2).

As the *N*-demethylation of NDMA to formaldehyde was detected in the peroxidase dependent system, it is clear that these enzymes catalyze the oxidation of NDMA simi-

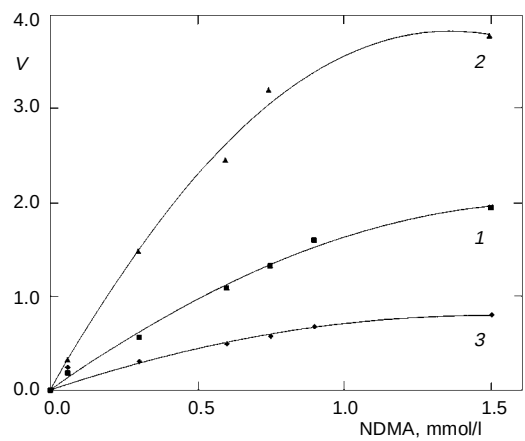


FIG. 1
Concentration dependence of NDMA demethylation rate *V* (nmol/min per mg protein) by tulip peroxidase A_1 (*1*), C (*2*) and E (*3*)

larly to the plant and animal cytochrome P450-systems, producing formaldehyde and methanediazonium ion[13,28,29].

More complex reactions of NMA oxidation were generated by cytochromes P450 (refs[13,30,31]). Therefore, the NMA oxidation catalyzed by tulip peroxidase was studied in more detail. The most efficient tulip peroxidase with respect to nitrosamines oxidation (repurified peroxidase C), was used in these studies. Special emphasis was laid on the identification and quantification not only of formaldehyde but also of other NMA metabolites formed by peroxidase.

The products of NMA oxidation generated by the tulip peroxidase were separated by HPLC. The metabolites were identified by comparison of their retention times with those of authentic standards. The unchanged NMA is not detected here as it remains bound on the SepPak cartridge. In addition to formation of formaldehyde, methyl-

TABLE II
The absorption maxima (nm) of tulip and horseradish peroxidases C

Source	Native peroxidase				Reduced peroxidase
	A	B	C	D	
Tulip	405	410	416	418	424
	498	578	520	554	
	640	640	558	585	
Horseradish	405	410	416	418	426
	497	580	524	549	
	641	642	556	583	

A, without H_2O_2; B, 50 nmol H_2O_2 added; C, 15 μmol H_2O_2 added; D, 15 μmol H_2O_2 added after 10 min.

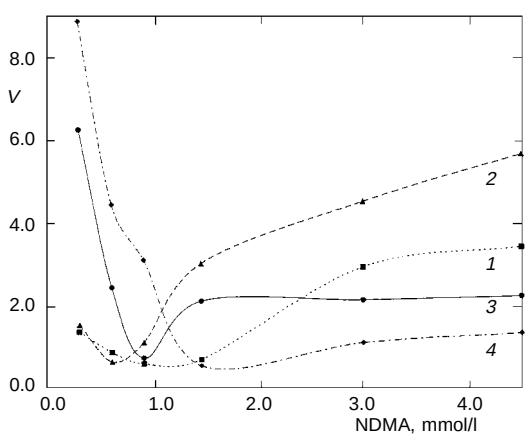


FIG. 2
Concentration dependence of NMA demethylation rate *V* (nmol/min per mg protein) by tulip peroxidase A_1 (*1*), C (*2*), D (*3*) and E (*4*)

TABLE III
Rate[a] of *N*-demethylation of NDMA and NMA by tulip peroxidases

Peroxidase	NMA		NDMA	
	0.3 μmol	3.0 μmol	0.3 μmol	3.0 μmol
A_1	0.057 ± 0.006[b]	0.122 ± 0.010	0.023 ± 0.002	0.064 ± 0.003
A_2	[c]	[c]	[c]	[c]
A_3	[c]	[c]	[c]	[c]
B	[c]	[c]	[c]	[c]
C	0.924 ± 0.050	0.240 ± 0.020	0.090 ± 0.008	0.136 ± 0.020
D	0.260 ± 0.020	0.082 ± 0.007	[c]	[c]
E	0.188 ± 0.020	0.042 ± 0.003	0.023 ± 0.002	0.034 ± 0.003

[a] nmol HCHO/min nmol peroxidase. [b] Means and standard deviations of three experiments. [c] Not detectable.

TABLE IV
Kinetic parameters of NDMA oxidation by tulip peroxidases

Peroxidase	K_m, mM	V_{max} nmol HCHO/min mol peroxidase
A_1	0.77 ± 0.006[a]	0.073 ± 0.005
C	0.42 ± 0.002	0.150 ± 0.030
E	0.60 ± 0.006	0.036 ± 0.003

[a] Means and standard deviations of three experiments.

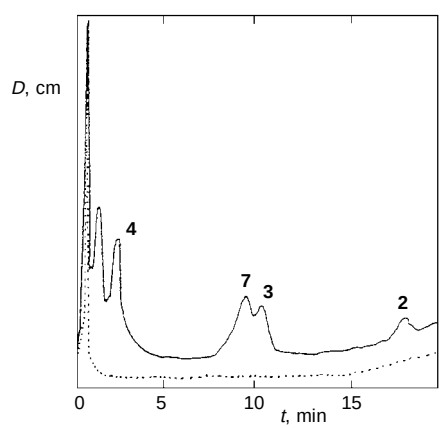
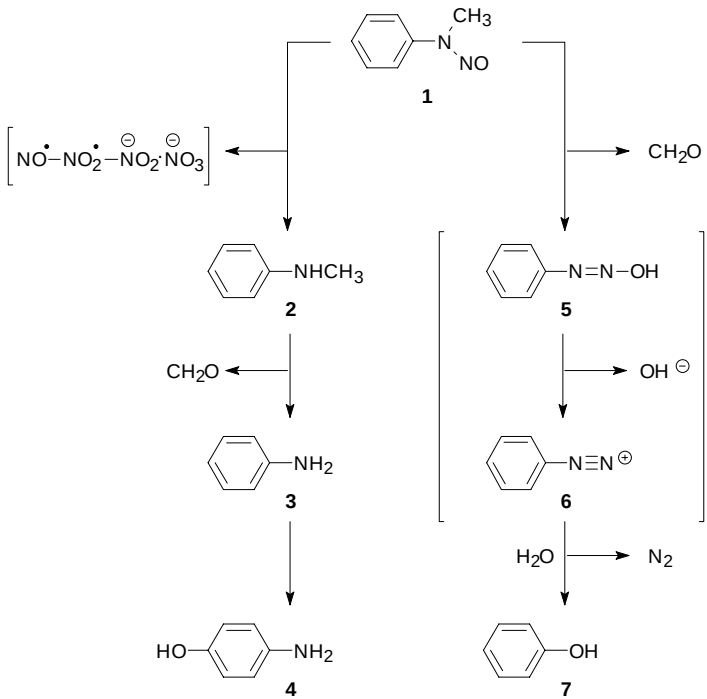


FIG. 3
HPLC profile of products formed in oxidation by a tulip peroxidase C. *D* is recorder deflexion. Numbers denote individual metabolites according Scheme 1

aniline, aniline and 4-aminophenol were found to be metabolites formed from NMA by this plant peroxidase (Fig. 3, Scheme 1). Phenol, which is the representative metabolite for the oxidative bioactivation pathway *via* the intermediary benzenediazonium ion (BDI) formation[30], was also determined and the yields were corrected for the about 20% recovery of phenol from BDI in the presence of the peroxidase. This compound is the main metabolite with about 5.3% relative to the NMA concentration (3 mM) within 10 min under the conditions used (Table V). *N*-Methylaniline and aniline, which are formed by denitrosation reactions as well as 4-aminophenol (the aniline hydroxylation metabolite) are minor products (Table V). On the basis of products formed we can postulate that tulip peroxidase C catalyzes both *N*-demethylation reaction (leading to formaldehyde and BDI) and denitrosation (Scheme 1).

DISCUSSION

It was suggested previously that peroxidases could be very important enzymes participating in oxidative reactions converting xenobiotics in plants[1–4]. These enzymes were assumed to be even more effective in metabolism of several xenobiotics in plants than



SCHEME 1

cytochromes P450 (refs[1,2,7]), which are the most important enzymes metabolizing xenobiotics in animals.

We previously described[12,13] the oxidation of both the nitrosamines studied in this work with isolated tulip cytochrome P450 including its kinetic characteristics. From the comparison of the kinetic data obtained for the oxidation of NDMA and NMA by tulip P450 and peroxidases, we can postulate that both nitrosamines are oxidized by tulip P450 *in vitro* more efficiently than by tulip peroxidases. The V_{max} value of NDMA oxidation by P450 is one order of magnitude higher than that obtained for its oxidation by tulip peroxidase C. Likewise, the K_m value obtained for tulip P450 is lower than that for peroxidase.

Surprisingly, the oxidation of NMA catalyzed by tulip peroxidases exhibits non-Michaelian kinetics. As yet we can only speculate about the mechanism of this peroxidase-mediated kinetics. From the detailed study of the pattern of products of the NMA oxidation (formaldehyde, methylaniline, aniline, 4-aminophenol and phenol), it follows that this xenobiotic is *N*-demethylated as well as denitrosated during the reaction (Table V, Fig. 3, Scheme 1). Phenol, which is the representative metabolite for the bioactivation pathway of NMA *via* intermediary BDI formation, is formed preferentially in comparison with the products formed by denitrosation (Table V). BDI, which was suggested to be the electrophilic species (formed from NMA) binding to DNA and proteins and, therefore, to be responsible for initiation of the NMA-induced carcinogenesis[32–34] is formed preferentially, too. Hence, this reactive intermediate could be bound to the peroxidase protein molecule forming the enzyme with different activity. This suggestion has, however, to be resolved in further studies.

Several products formed from NMA by tulip peroxidase are identical with those formed by tulip P450 (4-aminophenol, aniline, *N*-methylaniline). Although these products are identical, the mechanisms of oxidation catalyzed by the two enzymes are supposed to be different. It is known that the mechanism of *N*-dealkylation of several

TABLE V
In vitro metabolites of NMA by the tulip peroxidase C

Metabolite	Yields, %[a]
4-Aminophenol	1.40 ± 0.11[b]
Phenol	5.2[c] ± 0.43
Aniline	0.82 ± 0.09
N-Methylaniline	0.32 ± 0.02

[a] Based on added NMA (3 mmol/l). [b] Means and standard deviations of three experiments. [c] Corrected for 20% recovery of phenol from peroxidase.

substrates by peroxidases is different from the *N*-dealkylation mechanism suggested for P450 (ref.[35]). *N*-Demethylation catalyzed by peroxidases results in the formation of free radical cation and iminium cation by sequential one-electron oxidations. The latter intermediate is hydrolyzed to formaldehyde and demethylated amine[35–36]. This mechanism has, however, not been confirmed for *N*-demethylation of NMA and NDMA as yet. The question remains to be resolved in further studies. Phenol as the product of NMA oxidation is formed only by peroxidase. As the present study is aimed at contribution to knowledge which from the two plant enzymes (participating in metabolism of xenobiotics in plants) is more effective in the oxidation of NMA, the above mentioned finding is of a great significance. There is no doubt that the kinetic characteristics of NMA and NDMA obtained from catalysis by tulip P450 (ref.[13]) and peroxidase (present paper) *in vitro* showed that P450 is more effective in oxidation than tulip peroxidase. However, the biological significance of both P450 and peroxidase-mediated oxidation for NMA metabolism in plants *in vivo* is unknown. The determination of NMA metabolites formed in tulip plants *in vivo* would resolve this question. Such study is planned for in future studies. From this point of view, phenol can serve as a marker for a peroxidase-mediated metabolism of NMA in the tulip plant.

This study was supported by grants of the Ministry of Education of the Czech Republic (VS96141) and Foundation for the Development of the Universities (1996/0275).

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