

Full Length Research Paper

Biodesulfurization of dibenzothiophene and its alkylated derivatives through the sulfur-specific pathway by the bacterium *RIP1-S81*

Ladan Rashidi^{1*}, Ghasemali Mohebbi², Jafar Towfighi darian¹, Behnam Rasekh²

¹Chemical Engineering Department, Tarbiat Modarres University, Tehran, P.O.BOX. 14115- 4838, Iran.

²Department for microbiology, Research Institute of Petroleum Industry, Tehran, P.O.BOX. 18745- 4163, Iran.

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RIP1-S81 is a new dibenzothiophene (DBT)-desulfurizing bacterium, which was isolated by Research Institute of Petroleum Industry in Iran. Resting cells and growing cells of *RIP1-S81* was able to convert alkylated dibenzothiophenes (Cx-DBTs) to hydroxybiphenyls such that they were almost stoichiometrically accumulated as the dead-end metabolites of Cx-DBTs desulfurization in the medium containing minimal salt (MSM) and nutrients. *RIP1-S81* could desulfurize up to 80% of 4,6-dimethyldibenzothiophene and 50% of methyldibenzothiophene in the MSM containing 40 mg/l of a sulfur source. The molecular structures of metabolites and the reduction of Cx-DBTs were analyzed using GC-MS and HPLC. The position of alkyl substitutes and the sulfur substrate affected desulfurization rates.

Key words: Biodesulfurization, dibenzothiophene, 4, 6-dimethyldibenzothiophene, 4-methyldibenzothiophene.

INTRODUCTION

All fossil fuels contain organic and inorganic sulfur compounds. The combustion of these materials leads to release sulfur oxides that contribute to acid rain and air pollution. Alkylated dibenzothiophenes (Cx-DBTs) have been found to be highly recalcitrant in removal by HDS treatment. The most refractive sulfur compounds in gas oil streams are the high molecular weight molecules that contain alkyl side chains in positions 4 and 6. These positions limit the access of the molecule to the active sites on the catalyst (Tanaka et al., 1996). Therefore, biodesulfurization has attracted attention as a complementary system to HDS in which microbes or their

enzymes are used as the catalysts to remove sulfur from petroleum fractions. Several bacteria capable of metabolizing DBT and its alkylated derivatives have been isolated previously. Bacterial strains *Bacillus subtilis* WU-S2B (Kirimura et al., 2001), *Peanibacillus* A11-2 (Onaka et al., 2001a) exhibited DBT-desulfurizing activity under high temperature conditions. Some mesophilic bacteria such as *Rhodococcus erythropolis* IGTS8 (Kayser et al., 1993) and *R. erythropolis* H -2 (Ohshiro et al., 1996), *R. erythropolis* KA2-5-1 (Onaka et al., 2001b) have been isolated and investigated to date. All of them desulfurize DBT through a sulfur specific pathway without reducing the energy content of the molecule as shown in Figure 1 (Kirimura et al., 2001). In this research, we investigated the desulfurization ability of a newly isolated bacterium, which was able to use Cx -DBTs as the sulfur sources in the medium containing minimal salt and nutrients. We compared the rate of desulfurization of 4, 6-DMDBT and 4-MDBT by resting and growing cells of this bacterium.

*Correspondence author. E-mail: ladan_rsh@myway.com; Fax number: 0098-021-8057007.

Abbreviations: Biodesulfurization or biocatalytic desulfurization; Cx -DBT, Alkyl dibenzothiophene; DBT, Dibenzothiophene; DBTO, Dibenzothiophene sulfoxide; DBTO₂, Dibenzothiophene sulfone; DBTs, Dibenzothiophenes; DCW, Dry cell weight; 4,6-DMDBT, 4,6-Dimethyl dibenzo-thiophene; *dsz*, Desulfurization; 2-HBP, 2- Hydroxy biphenyl; 2-HDMBP, 2-Hydroxy 3,3 -dimethyl biphenyl; HDS, Hydrodesulfurization; 2-HMBP, 2-Hydroxy 3- methyl biphenyl; 2 -HMBP, 2-Hydroxy 3 -methyl biphenyl; and 4-MDBT, 4-Methyl dibenzothiophene.

MATERIALS AND METHODS

Chemicals

DBT and 2-HBP were of high quality purchased from Merck. 4, 6-DMDBT and 4-MDBT were purchased from Aldrich-Sigma. All other

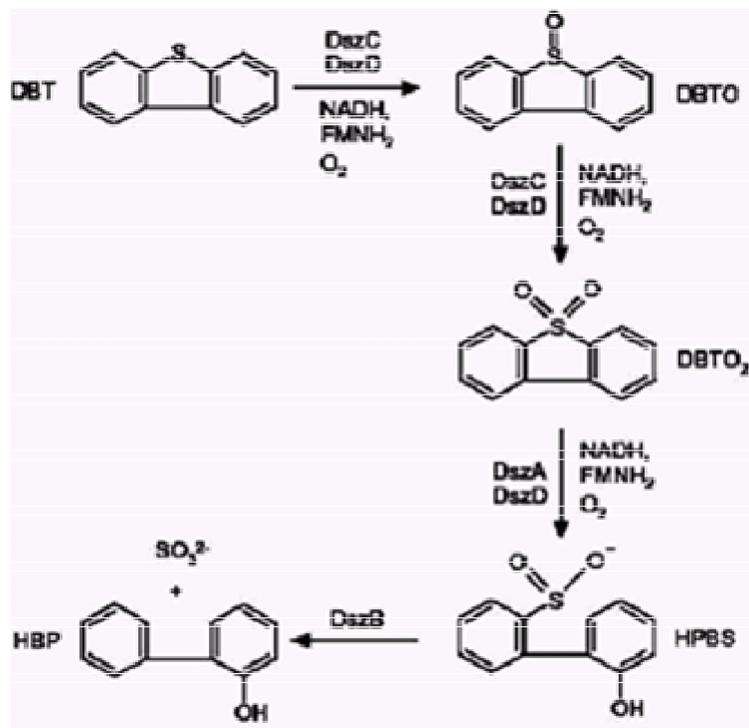


Figure 1. Proposed pathway of DBT- desulfurizing by several bacteria including *RIPI-S81*.

commercially available chemicals were of analytical grade prepared.

Bacterial and culture medium

This bacterium is a coccobacil, Gram positive, non-motile, and strictly aerobic. The other characteristics of this bacterium are under investigation. In order to prepare one liter of the sulfur-free medium for the growth of *RIPI-S81* the following compounds were dissolved in 850 ml distilled water: KH₂PO₄ (6 g), Na₂HPO₄ (4 g), NH₄NO₃ (1.2 g) and 2g of C₇H₅NaO₂ (pH 7.08). The second solution comprised of: MgCl₂ · 6H₂O (0.75 g), MnCl₂ · 4H₂O (0.004 g), CaCl₂ · 2H₂O (0.001 g), and 0.001 g of FeCl₃ in 150 ml distilled water. After autoclaving, the solutions were mixed and then used. N, N-dimethylformamide (DMF) solution of DBT, 4, 6-DMDBT or 4-MDBT (about 2%, w/w) was also prepared.

Analytical procedures

Growth and dry cell weight was measured by using a spectrophotometer (model UV mini 1240 CE, Shimadzu) at 660 nm. The course of bacterial growth aliquots of culture were removed and acidified to pH 2 by 1 N HCL, followed by extraction with ethyl acetate. A portion of the ethyl acetate layer was used for HPLC analysis (model waters-600E, type LC-9A; Shimadzu, Japan). The molecular structures of metabolites were analyzed by a Varian model 3400 gas chromatograph (a DB-5 fused-silica capillary column 25 m, 0.25 mm, 0.25 μm) connected to model Saturn ion-trap mass spectrometer (GC-MS). The accumulation of phenolic compounds was determined by the Gibb's assay (Kayser et al., 1993).

Desulfurization of DBT and its alkylated derivatives

RIPI-S81 was precultivated in 40 ml of the minimal salt (MSM) supplemented with 40 mg/l of DBT as a sole sulfur source, at 30°C for 4 days and then it was inoculated into 4 l of the same and new prepared medium. After mixing, the culture bearing medium was distributed in 80 flasks, each 50 ml volume in a 100 ml flask and then 4-MDBT or 4,6-DMDBT solution (40 mg/l) was added to each flask as a sole sulfur source and incubated at 30° with shaking (120 rpm). 2 flasks with the same medium not inoculated were incubated under the same condition for control. At the defined time intervals, two or three of samples were used for pH, optical density (OD₆₆₀), Gibb's assay (OD₆₁₀) measurements and then produced metabolites extracted with ethyl acetate for HPLC and GC-MS analysis.

Resting cells reactions

RIPI-S81 was precultivated in 400 ml of the medium (MSM) supplemented with 40 mg/l of DBT solution as a sole sulfur source at 30°C for 4 days. At the late logarithmic phase, unused DBT particles were isolated from samples by centrifugation at 1,000 rpm for 1 min, then cells were harvested by centrifugation at 4,000 rpm for 8 min. The harvested cells were washed twice with 0.1 M of potassium phosphate buffer (pH 7.08) and suspended in the same buffer. The OD₆₆₀ of the cell suspension was adjusted to 30. One OD₆₆₀ unit corresponded to 0.5773 g of cells (dry cell weight) per liter. Resting cell reaction was carried out in the test tubes (2 ml) containing 0.5 ml of the cell suspension and 200 mg/l of each of the sulfur substrate. The reaction was performed at 30°C with reciprocal shaking at 225 rpm for 24 h and stopped by the acidification, then metabolites were extracted by ethyl acetate for

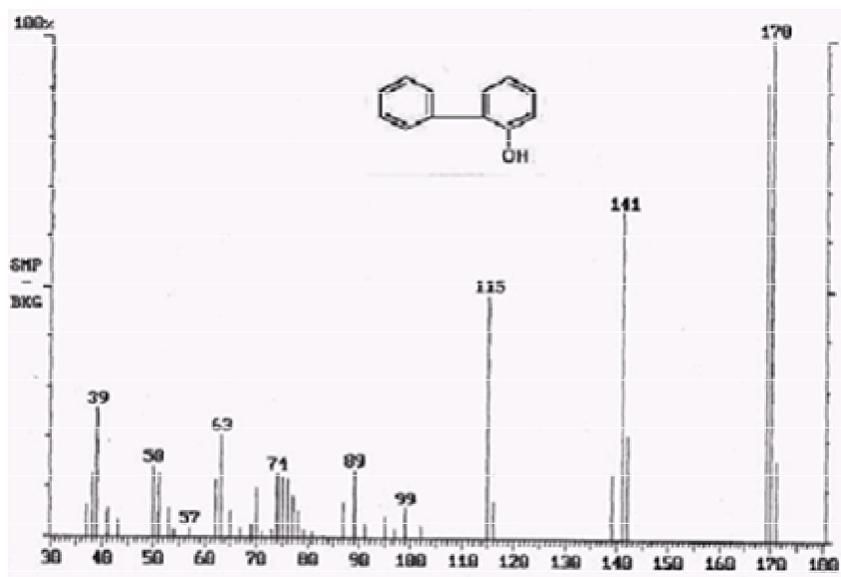


Figure 2. GC-MS spectrum of the DBT final metabolite with strain *RIP1-S81*. This metabolite was identified as 2-HBP.

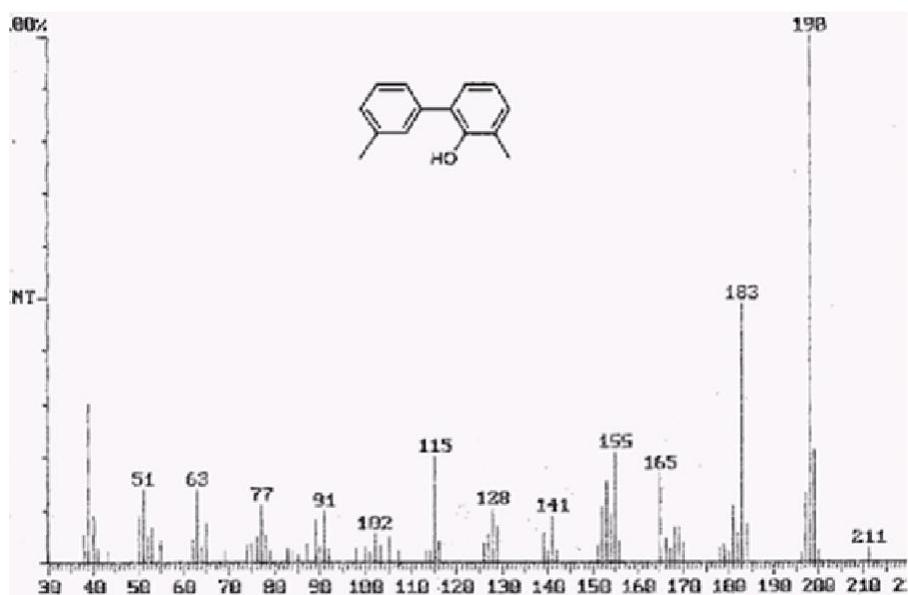


Figure 3. GC-MS spectrum of the 4,6-DMDBT final metabolite with strain *RIP1-S81*. This metabolite was identified 2-HDMBP.

analyzing.

RESULTS AND DISCUSSION

The desulfurization activities were studied in medium containing DBT as a sole sulfur source. *RIP1-S81* could desulfurize 40 mg/l of DBT at 30°C for 4 days. GC-MS analysis of the culture extracts confirmed that the end

product of DBT desulfurization pathway was 2-HBP. The detected metabolite of DBT desulfurization by *RIP1-S81* had a molecular mass ion (m/z) of 170 and its mass spectrum corresponded to the 2-HBP (Figure 2). This strain grew well in the medium containing of 40 mg/l of 4, 6-DMDBT as a sole sulfur source. The microbial metabolite of *RIP1-S81* on 4, 6-DMDBT was identified

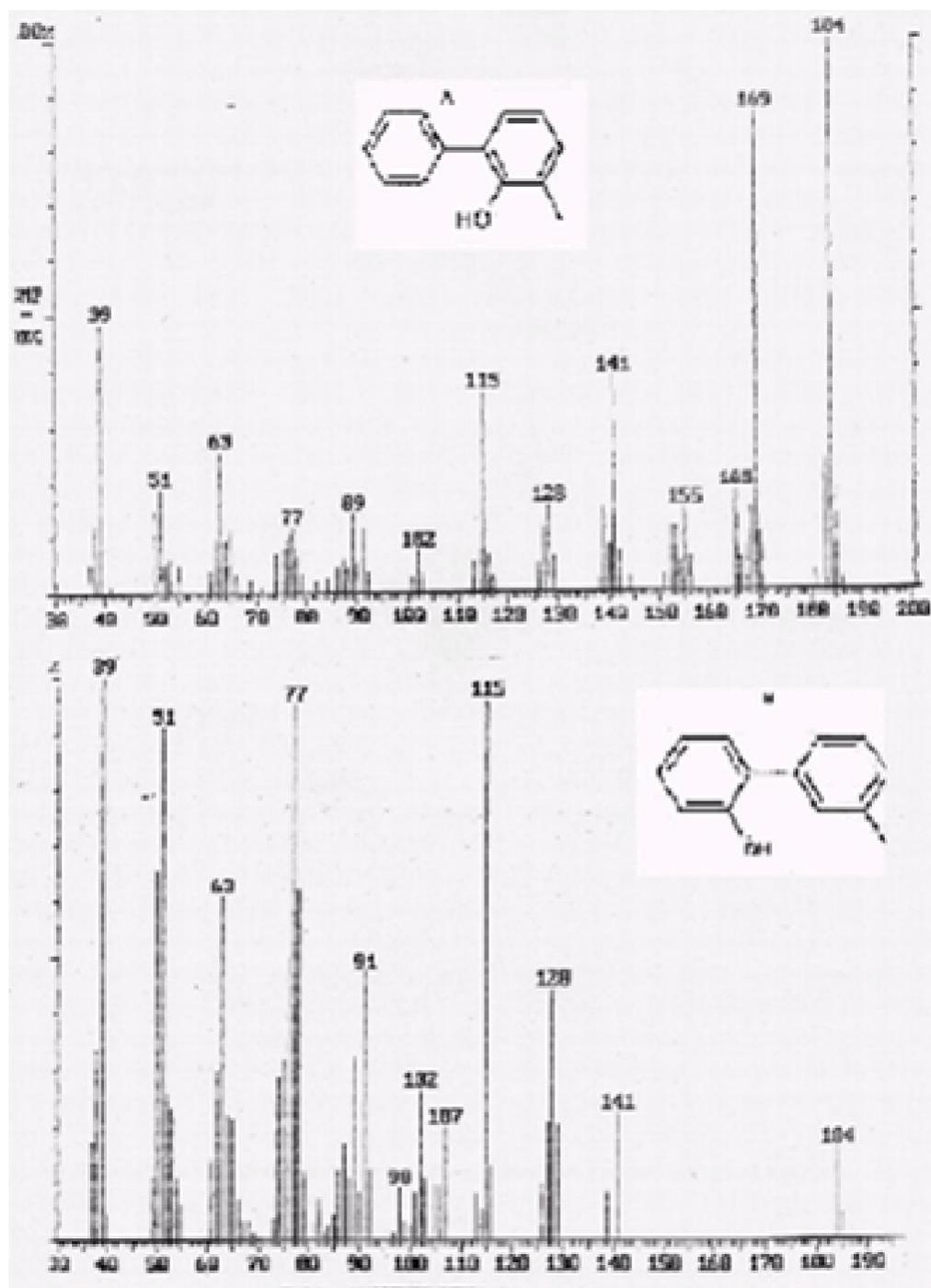


Figure 4. GC-MS spectrum of the final metabolites with strain *RIP1-S81*. These metabolites were identified as, A: 2-hydroxy-3-methyl-biphenyl & B: 2-hydroxy-3-methyl-biphenyl (2-HMBP).

using GC-MS analysis having a mass ion at m/z 198 was in accordance with the molecular mass of monohydroxy-dimethyl-biphenyl. Its mass spectrum corresponded to the 2-hydroxy-3, 3'-dimethylbiphenyl (2-HDMBP) as shown in Figure 3. Growing cells of *RIP1-S81* could also desulfurize 40 mg/l of 4-MDBT in the medium as a sole

sulfur source. Using GC-MS analysis, two metabolites were identified with mass ion at m/z 184 which were in accordance with the molecular mass of monohydroxy-methyl-biphenyl (Figure 4). According to the methods of Onaka et al. (2000), the metabolites of 4-MDBT were proposed to be 2-hydroxy-3-methyl-biphenyl and

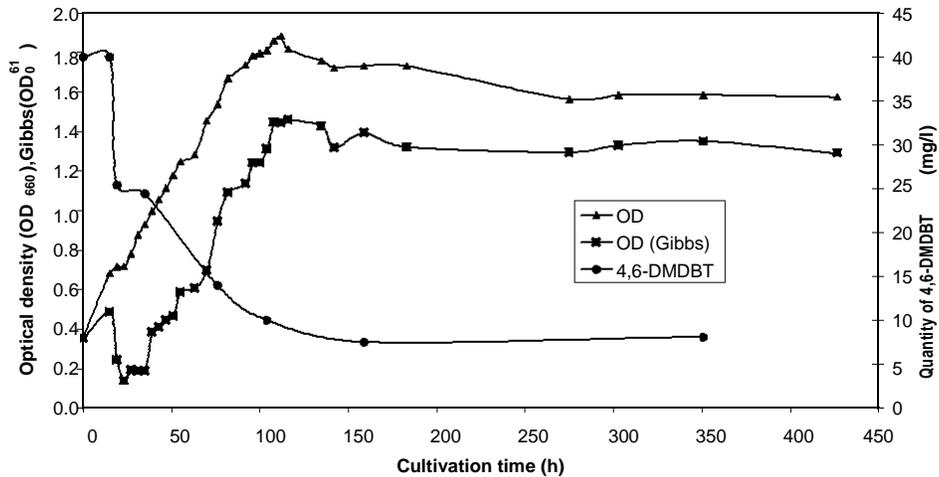


Figure 5. Time course of 4, 6-DMDBT desulfurization at 30°C by growing cells of *R1PI-S81*.

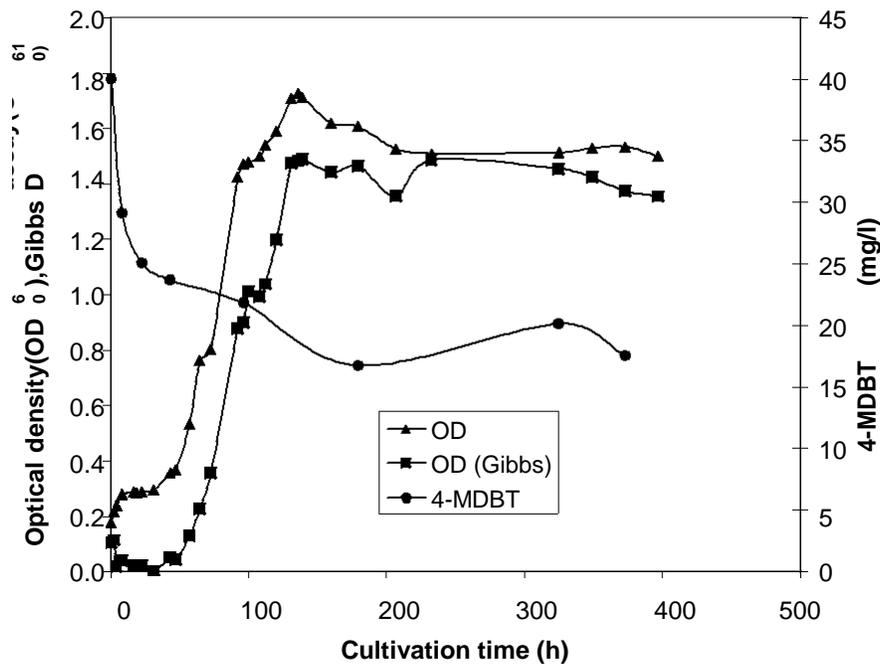


Figure 6. Time course of 4-MDBT desulfurization at 30°C by growing cells of *R1PI-S81*.

2-hydroxy-3-methyl-biphenyl. Resting cells of *R1PI-S81* could also desulfurize 200 mg/l of each of the sulfur sources and produce these metabolites.

R1PI-S81 grew in the medium containing 4, 6-DMDBT as the sulfur sources. In Figure 5 the growth of *R1PI-S81* is shown. The strain showed maximum growth (about 1.884 units of optical density at 660 nm) at 112 h. In addition, in Figure 5, the quantity of accumulating phenolic compounds was shown by Gibb's assay. The phenolic compounds formation reached a maximum after 116 h. HPLC analysis of the culture extracts showed the decrease of 4,6-DMDBT with time (Figure 5). The

maximum reduction of 4, 6-DMDBT was reached after 104 to 112 h during the growth phase of the bacterium. 2-HDMBP increase was almost equal to 4, 6-DMDBT reduction in the culture and did not decrease during stationary phase. It shows that this bacterium does not use 4, 6-DMDBT as a substrate.

This strain grew in a MSM containing 4-MDBT as a sole sulfur source. The maximum growth of *R1PI-S81* was about 1.727 units of optical density at 660 nm for 136 h. Growth of *R1PI-S81* and the quantities of accumulated phenolic compounds (as Gibb's assay results) in the cultures were shown in Figure 6. The maximum reduction

of 4-MDBT was obtained during 110 to 136 h of growth phase. Desulfurization of 4-MDBT was slower and weaker than other sulfur compounds used in this study. The results indicated that the manner of attack on these compounds might be affected by the position of alkyl substituents and the sulfur substrate. Therefore, it was found that the produced 2-hydroxy-3-methylbiphenyl (2-HMBP) was higher than 2-hydroxy-3'-methylbiphenyl through the growth phase of *RIP1-S81*. These metabolites are not used by the strain as substrate. The growth rate of *RIP1-S81* using 4, 6-DMDBT is 0.0149/h and for 4-MDBT it is 0.0159/h.

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