Relationship between Uptake of *p*-Hydroxybenzoic Acid Esters by *Escherichia coli* and Antibacterial Activity[1)]

Masahiro FUKAHORI,*[,a] Sakae AKATSU,[a] Hiroshi SATO,[a] and Toshihisa YOTSUYANAGI[b]

Central Research Laboratories, Zeria Pharmaceutical Co., Ltd.,[a] 2512–1, Oshikiri, Konan-machi, Osato-gun, Saitama 360–01, Japan and Faculty of Pharmaceutical Sciences, Nagoya City University,[b] Mizuho-ku, Nagoya 467, Japan.
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The relationship between the uptake and antimicrobial activity of *p*-hydroxybenzoic acid esters (parabens) was studied using *Escherichia coli*. The uptake into bacterial cells and the antibacterial activity of parabens were logarithmically proportional to the carbon number of the alkyl group from methyl to butyl paraben. The free energy change for the transfer of the methylene group of parabens from the aqueous to the cell phase was less than that obtained from the *n*-hexane and *n*-octanol–water partition systems. This demonstrates that the hydrophilicity of the cells is larger than *n*-hexane and *n*-octanol. The uptake of hydrophobic ethyl benzoate was less than that of the more hydrophilic butyl paraben possessing a phenolic hydroxyl group. Parabens may thus be incorporated into cells by both hydrophobic and hydrophilic interactions. The apparent concentration of parabens in the bacterial cells required to produce the same antibacterial activity decreased logarithmically with an increasing carbon number of the alkyl group. The dependence of the antibacterial activity of parabens on the alkyl chain length may thus be concluded to be due to the alkyl group, not only for uptake into bacterial cells but also for accumulation or concentration on biological receptors after incorporation into the cells.

Key words hydroxybenzoic acid ester; antibacterial activity; uptake; *Escherichia coli*; methylene group contribution; partition coefficient

The physicochemical parameters of drugs in an aqueous solution, such as solubility, partition behavior, self-association and adsorption, are essential for assessing their biological activity or availability. *p*-Hydroxybenzoic acid esters (parabens) are widely used as antimicrobial preservatives for aqueous based pharmaceuticals and cosmetics. The partition of parabens between non-ionic surfactant micellar and aqueous phases and the self-association of parabens in an aqueous solution were previously found to be dependent on the carbon number of the alkyl group.[1-3)] The antimicrobial activity of parabens is rapidly enhanced according to the carbon number of the alkyl group.[4-6)] Changing, in self-association with alkyl chain length, may thus cause the antimicrobial activity to depend on the alkyl chain length. Preservative molecules are considered to be adsorbed on cell walls or incorporated in the protoplasm of microorganisms by van der Waals force, hydrogen bonding and ionic bonding.[7)] The antimicrobial activity of parabens with varied alkyl chain length may be related to the magnitude of paraben uptake into the cell membrane and cytoplasm of microorganisms. Little has been reported on the uptake of parabens into microorganism cells.

To clarify the relationship between the antimicrobial activity of parabens and their uptake into microorganisms, parabens from methyl to butyl ester and *Escherichia coli* were used. The uptake of ethyl benzoate derivatives into bacterial cells was also examined.

Experimental

Materials Methyl, ethyl, propyl and butyl *p*-hydroxybenzoate (MP, EP, PP and BP, respectively, Ueno Pharmaceutical Co., Tokyo) were of JP XII grade. Ethyl *m*-hydroxybenzoate, ethyl *o*-hydroxybenzoate, ethyl benzoate, *n*-octanol and *n*-hexane (Wako Pure Chemical Industries, Ltd.) were of analytical reagent grade. All other reagents and solvents were of analytical reagent grade.

Apparent Partition Coefficient Determination between *n*-Octanol or *n*-Hexane and Water Parabens and ethyl benzoate derivatives were dissolved in *n*-octanol and *n*-hexane (BP, ethyl *o*-hydroxybenzoate, ethyl benzoate) or water (MP, EP, PP, ethyl *m*-hydroxybenzoate). One ml *n*-octanol containing parabens or ethyl benzoate derivatives ($\simeq 1.5 \times 10^{-2}$ M) and 9.0 ml water, 5 ml *n*-hexane containing BP or ethyl benzoate derivatives ($\simeq 1.5 \times 10^{-2}$ M) and 5 ml water or 5 ml *n*-hexane and 5 ml water containing parabens or ethyl *m*-hydroxybenzoate ($\simeq 2.0 \times 10^{-3}$ M) were charged into a glass-stoppered centrifuge tube. After being shaken at 25 °C for 24 h and centrifuged ($1600 \times g$, 5 min), the concentrations of parabens and ethyl benzoate derivatives in the upper *n*-octanol or *n*-hexane and lower aqueous phase were determined by HPLC under the following conditions: reversed-phase column (Inertsil ODS-2 4.6 × 150 mm, GL Sciences Inc., Tokyo); detection (UV at absorption maxima); mobile phase (methanol : water = 3 : 2, v/v); flow rate of 1.0 ml/min; temperature at 40 °C.

Bacterial Cell Culture and Preparation of Cell Suspension *Escherichia coli* NIHJ (*E. coli*) was incubated at 37 °C for 18 h in soybean-casein digest agar or broth, adjusted at pH 7.0 and sterilized at 120 °C for 15 min. Bacterial cells aseptically scraped from the agar medium were suspended in sterilized distilled water and diluted to a prescribed cell concentration to be used as an inoculated solution. Bacterial cells in the broth medium were aseptically collected and washed twice with distilled water by centrifugation ($1600 \times g$, 20 min) at 25 °C for the uptake measurement of drugs.

Cell Number and Cell Volume Determinations The cell numbers in the cell suspension were determined by the plate count method using soybean-casein digest agar, adjusted at pH 7.0 and sterilized at 120 °C for 15 min. The cell suspensions, diluted stepwise with sterilized physiological saline (1.0 ml), were spread in an agar medium. After the agar plates were incubated at 37 °C for 24 h, the cell colonies were counted according to the cell numbers in each suspension.

The packed cell volume of *E. coli* was determined using a plain hematocrit capillary tube (1.5 × 75 mm, Terumo Co.) and a hematocrit centrifuge (model RC-24B, Tomy Seiko Co., Tokyo). The collected cells were suspended in distilled water, then poured into the capillary tube. After the tube was centrifuged (11500 rpm) at room temperature for 5 min, the packed cell volume was determined from the height of the precipitated cells.

Determination of Minimum Bactericidal Concentration (MBC) of Parabens The diluted cell suspension, 0.1 ml, was inoculated in a 4.9 ml aqueous paraben solution, where the cell number was always set at 6.0×10^5 cells/ml. The paraben concentration in the solution was adjusted to a multiple of 5.79×10^{-4} M of MP, 2.90×10^{-4} M of EP, 1.45×10^{-4} M of PP and 0.724×10^{-4} M of BP to 116×10^{-4} M of MP,

* To whom correspondence should be addressed.

58.4×10^{-4} M of EP, 20.3×10^{-4} M of PP and 7.24×10^{-4} M of BP, respectively. After being kept at 25 °C for 6 d, the paraben solution with the cells (0.1 ml) was inoculated in *ca.* 5 ml soybean-casein digest broth with lecithin and polysorbate 80, adjusted at pH 7.0 and sterilized at 120 °C for 15 min. The MBC of parabens was determined after no bacterial growth in the broth was evident following inoculation at 37 °C for 24 h.

Uptake Measurement of Parabens and Ethyl Benzoate Derivatives in Bacterial Cells The collected *E. coli* cells were suspended in aqueous paraben or ethyl benzoate derivative solutions. A glass-stoppered centrifuge tube (volume of *ca.* 15 ml) packed with 2 ml of the cell suspension was shaken with a flask shaker (120 rpm, model TB-12T, Takasaki Kagaku Kikai Co., Saitama) at 25 °C for 60 min. Concentrations of the drugs in the cell suspension were adjusted to 7.24×10^{-4} M, corresponding to *ca.* 0.01% (w/v) as *p*-hydroxybenzoic acid. Paraben uptake was also examined at the MBC. Packed cell volume and cell numbers in the cell suspensions were 0.050 ml/ml and 1.1×10^{10} cells/ml, respectively. Following centrifugation ($1600 \times g$, 20 min) at 25 °C, the concentrations of the drugs in the supernatant were determined by HPLC. No difference in the uptake of the drugs in the solutions prepared by distilled water or physiological saline was observed under the experimental conditions. A difference in drug concentrations between the supernatant and cell suspension was considered to be due to the uptake of the drugs into the cells. The ratio of the amount of the drug incorporated into *E. coli* cells to the packed cell volume was regarded as the apparent concentration of drug in the cells, C_m (mol/l of cells), determined by Eq. 1:

$$C_m = D_m/V_m = (D_t - C_w(V_t - V_m))/V_m \quad (1)$$

where D_m and D_t are the amounts of a drug in the *E. coli* cells and in the cell suspension, respectively, V_m and V_t are the volumes of the packed cells and the cell suspension, respectively, and C_w is the concentration of the drug in the supernatant in the cell suspension.

Uptake Measurement of BP in Cell Wall and Cytoplasm Collected cells suspended in distilled water (1.8×10^{11} cells/ml) were disintegrated ultrasonically for 60 s using a probe-type sonicater (model 50, Yamato Scientific Co., Tokyo) on an ice bath. No intact cells could be seen microscopically in the disintegrated cell suspension. A BP solution of 6 ml (7.24×10^{-4} M) with disintegrated cells (corresponding to 1.1×10^{10} intact cells/ml) was shaken at 25 °C for 60 min in the same manner as above, followed by ultracentrifugation ($100000 \times g$, 30 min) with an ultracentrifuge (model L8-55, Beckman) at 25 °C. Two ml of the supernatant, including mainly cytoplasmic constituents, was poured into an ultrafiltration unit (Ultrafree CL-LCC, Nihon Millipore Co.), followed by centrifugal filtration at 25 °C. The first ultrafiltrate and remainder were rejected to avoid the possible adsorption of BP to the ultrafiltration unit. Two ml of methanol were added to the precipitate, including mainly cell walls and membranes, and the mixture was vigorously shaken. Following centrifugation ($1600 \times g$, 20 min) at 25 °C, the supernatant methanol was filtered through a membrane filter (pore size 0.2 μm). The BP concentrations in the ultrafiltrate, and the aqueous and methanol supernatant were determined by HPLC. The BP in the ultrafiltrate was regarded as unbound BP in an aqueous phase, and that in the methanol supernatant and the difference of BP between the aqueous supernatant and ultrafiltrate were regarded as the incorporated BP in the cell wall or membranes and cytoplasmic constituents, respectively.

Results and Discussion

Apparent Partition Coefficients in a *n*-Hexane or *n*-Octanol–Water System Table 1 shows the apparent partition coefficients of parabens and ethyl benzoate derivatives between *n*-hexane (K'_{hw}) or *n*-octanol (K'_{ow}) and water. The K'_{ow} of the drugs was greater than the K'_{hw}, and both values increased with the carbon number of the alkyl group of parabens.

MBC of Parabens Figure 1 shows the relationship between the minimum bactericidal concentration of parabens, MBC, against *E. coli* and the carbon number of the alkyl group of parabens. The MBC was 104—116×10^{-4} M of MP, 34.8—49.2×10^{-4} M of EP, 15.9—17.4×10^{-4} M of PP and 5.07—6.52×10^{-4} M of BP. The logarithms of the MBC were found to decrease linearly with an increasing carbon number of the alkyl group of parabens.

Table 1. Apparent Partition Coefficients of Parabens and Ethyl Benzoate Derivatives between *n*-Hexane (K'_{hw}) or *n*-Octanol (K'_{ow}) and Water at 25 °C

Compound	Apparent partition coefficient	
	K'_{hw}	K'_{ow}
MP	0.0292	80.9
EP	0.130	263
PP	0.547	952
BP	2.63	3350
Ethyl benzoate	302	480
Ethyl *o*-hydroxybenzoate	275	1021
Ethyl *m*-hydroxybenzoate	0.265	242

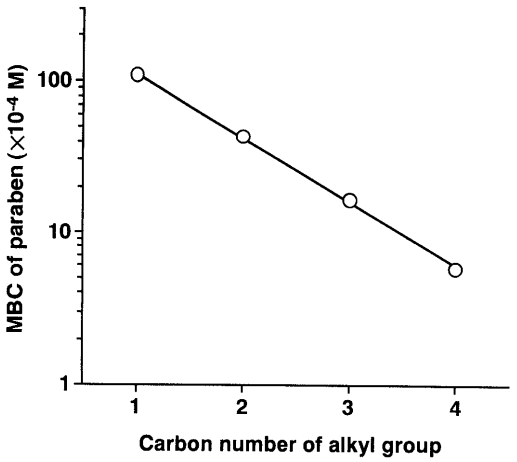

Fig. 1. Relationship between the MBC of Parabens against *E. coli* and the Carbon Number of the Alkyl Group of Parabens

Each point is the mean of three measurements.

A well-defined minimum inhibitory concentration (MIC) of parabens against various microorganisms was rapidly enhanced with a decrease in alkyl chain length.[4-6] However, the MIC may be influenced by interactions between antimicrobial agents and the components in the medium, since MIC is measured in a medium with microorganisms. The antibacterial activity of parabens in aqueous based pharmaceuticals could be predicted exactly by MBC measured in an aqueous solution. A good correlation of logarithms of the MBC and the carbon number demonstrates that the antibacterial activity is related to the hydrophobicity of parabens in analogy with a self-association ability which depends on alkyl chain length.[3]

Uptake of Parabens by Bacterial Cells Table 2 shows the paraben concentrations in the supernatant phase in an *E. coli* suspension after shaking for 60 min. The concentration of parabens decreased with an increasing carbon number of the alkyl group. No change in the BP concentration was observed by shaking for 15—120 min (data not shown). A decrease in parabens in the supernatant may thus be regarded as due to uptake into the cells. The apparent concentrations of parabens in the *E. coli* cells, C_m, determined according to Eq. 1, were shown to

Table 2. Paraben Concentrations in Supernatant of *E. coli* Suspension (C_w)[a]

Paraben	C_w[b] (M × 10^4)	Ratio[c]
MP	6.86±0.10	0.948
EP	6.55±0.22	0.905
PP	5.21±0.02	0.720
BP	3.09±0.24	0.427

a) Cell suspension with 0.050 ml/ml of the cells and 7.24×10^{-4} M of parabens. *b*) Each value is the mean and S.D. ($n=3$). *c*) Against initial paraben concentration.

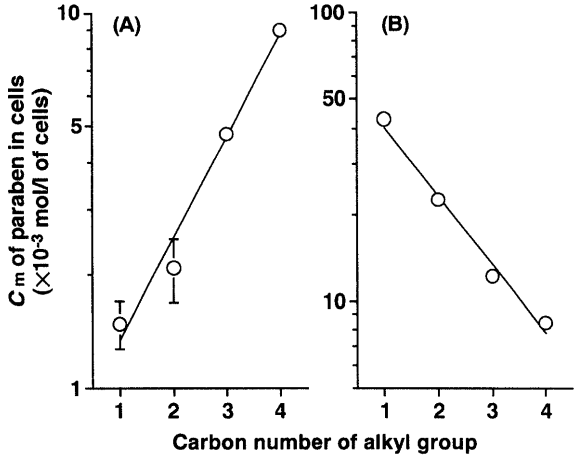

Fig. 2. Relationship between the C_m in *E. coli* Cells and the Carbon Number of the Alkyl Group of Parabens at (A) 7.24×10^{-4} M and (B) MBC of Parabens in a Cell Suspension

Each point is the mean±S.D. ($n=3$).

Table 3. Distribution of BP in *E. coli* Suspension with Disintegrated Cells

Fraction	Amount of BP[a] (mol × 10^4)	Ratio[b]
Disintegrated cells	4.43±0.05	1.00
Insoluble fraction	2.15±0.33	0.485
Soluble fraction	2.28±0.28	0.515
Aqueous phase	2.81±0.05	

a) BP per one liter of cell suspension with 0.050 ml/ml of the cells and 7.24×10^{-4} M of BP. Each value is the mean and S.D. ($n=3$). *b*) Against disintegrated cells.

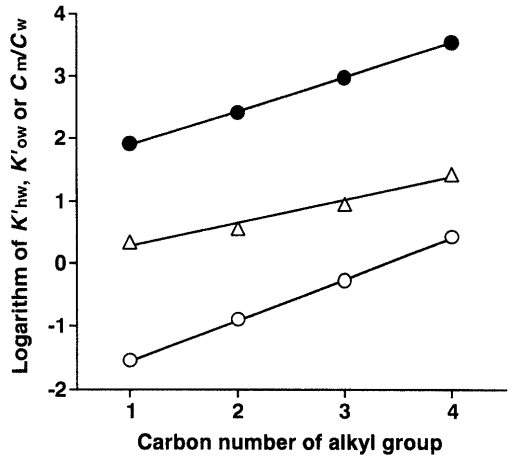

Fig. 3. Relationship between Logarithms of K'_{hw}, K'_{ow} or C_m/C_w and the Carbon Number of the Alkyl Group of Parabens

Keys: ○, K'_{hw}; ●, K'_{ow}; △, C_m/C_w.

be logarithmically proportional to the carbon number of the alkyl group, as shown in Fig. 2A. A bacterial cell surface consists of an outer cell wall, including peptidoglycan and lipopolysaccharide, an inner cytoplasmic membrane, including phospholipids and lipoprotein high in lipid content, and cytoplasm containing hydrophobic constituents.[8] A good correlation of the C_m and carbon number suggests that the extent of paraben uptaken into bacterial cells is related to the antibacterial activity. The paraben uptake in the bacterial cells may thus be regarded as a hydrophobic interaction between parabens and lipophilic constituents in the cells.

When the same bactericidal activity for *E. coli* was observed (at the MBC), the C_m of parabens in the cells decreased logarithmically with increasing carbon number of the alkyl group, as shown in Fig. 2B. The bactericidal mechanism of parabens was reported to be the destruction of the cell membrane,[6,9] the synthetic inhibition of intracellular DNA and RNA[6] and the uptake inhibition of amino acids.[10] Possible targets for the antimicrobial action of parabens fall into three groups: the cellular membrane, genetic constituents and enzymes in cytoplasm. About the same quantities of BP were incorporated into the insoluble fraction, including the outer cell wall and membrane, and the soluble fraction, including the inner cytoplasm, as shown in Table 3. These results indicate that the BP is accumulated in cytoplasmic components after permeation through the cell wall and membrane of intact cells. Most paraben molecules incorporated into the cells may be adsorbed and dissolved in the lipophilic regions of cell walls, membranes and cytoplasm. The dependence of C_m at the MBC on the alkyl chain length thus suggests that the hydrophobicity of parabens contributes to its accumulation or concentration on biological receptors from lipophilic regions, including a great quantity of parabens after incorporation into the cells.

Methylene Group Contribution to Uptake of Parabens by Bacterial Cells The ratio of the apparent concentrations of parabens in *E. coli* cells to the concentrations in the aqueous phase in the cell suspension, C_m/C_w, corresponds to an apparent partition coefficient of parabens between *E. coli* cells and the aqueous phase. Figure 3 shows the relationship between the logarithms of K'_{hw}, K'_{ow} or C_m/C_w and the carbon number of the alkyl group of parabens. Logarithms of K'_{hw}, K'_{ow} or C_m/C_w increased linearly with the carbon number of the alkyl group.

The logarithm of the partition coefficient is proportional to the free energy change for the transfer of a solute between two phases, and the slope, $\log F$, indicates the free energy change per a methylene group. The free energy change for the transfer of the methylene group from an aqueous to solvent phase, $\Delta(\Delta G)$, is defined as:[11]

$$\Delta(\Delta G) = -2.303 \boldsymbol{RT} \cdot \log F \quad (2)$$

where $\boldsymbol{R}$ is the gas constant and T the thermodynamic temperature.

Table 4 shows the $\Delta(\Delta G)$ determined according to Eq.

Table 4. Methylene Group Contribution to Partitioning of Parabens between Organic Solvents or Bacterial Cells and Water at 25 °C

Solvent or bacteria	$\Delta(\Delta G)$[a] (cal/mol)
n-Hexane	886
n-Octanol	738
E. coli	504

a) Free energy change for transfer of a methylene group calculated according to Eq. 2.

2 for the distribution of parabens between organic solvents or *E. coli* cells and the aqueous phase. The $\Delta(\Delta G)$ obtained from *n*-hexane and *n*-octanol systems agreed well with that in the literature (880 and 685 cal/mol, respectively).[12] The polarity of the uptake site in *E. coli* cells is considered to be larger than *n*-hexane or *n*-octanol, because the $\Delta(\Delta G)$ obtained from the cell system (corresponding to 504 cal/mol) is less than that of the solvents, and similar to that of 2-butanol or 3-pentanone, having 548 or 452 cal/mol.[12] The hydrophobicity of a bacterial cell surface was reported to vary with bacterial strains,[13,14] possibly due to differences in the composition of hydrophilic constituents in bacterial cells, such as peptidoglycan and protein. The $\Delta(\Delta G)$ obtained from bacterial systems thus appears to vary with the strains. The susceptibility of bacteria to quaternary ammonium salts depends on the hydrophobicity of bacterial cells.[14,15] Similarly, the extent of an increase in antibacterial activity caused by an increasing alkyl chain length[4-6] may be dependent on the polarity of the constituents in the bacterial cells.

Relationship between Uptake by Bacterial Cells and Apparent Partition Coefficients Figure 4 shows plots of the C_m of parabens and ethyl benzoate derivatives in *E. coli* cells against the apparent partition coefficients of these drugs between *n*-hexane (K'_{hw}) or *n*-octanol (K'_{ow}) and water. The C_m of parabens increased with increasing K'_{hw} and K'_{ow} (solid line in Fig. 4). Ethyl *m*-hydroxybenzoate, whose hydrophobicity was slightly larger than EP owing to the substituent position,[16] behaved in analogy with the *para* substituent EP. The relationship between C_m and K'_{hw} or K'_{ow} obtained from ethyl *o*-hydroxybenzoate and ethyl benzoate was different from that of the parabens.

Hydrophobic interactions contribute to the distribution of drugs in *n*-hexane, whereas not only hydrophobic interactions but also hydrogen bonding contributes to that in *n*-octanol. Ethyl *o*-hydroxybenzoate and ethyl benzoate possess great hydrophobicity due to intramolecular hydrogen bonding between the phenolic hydroxyl group and carbonyl oxygen atom and to the deletion of the phenolic hydroxyl group, respectively. Both drugs thus indicated much greater K'_{hw} than parabens and ethyl *m*-hydroxybenzoate. However, the C_m of hydrophobic ethyl *o*-hydroxybenzoate and ethyl benzoate was about the same as that of the more hydrophilic BP and PP having a phenolic hydroxyl group, respectively. These suggest

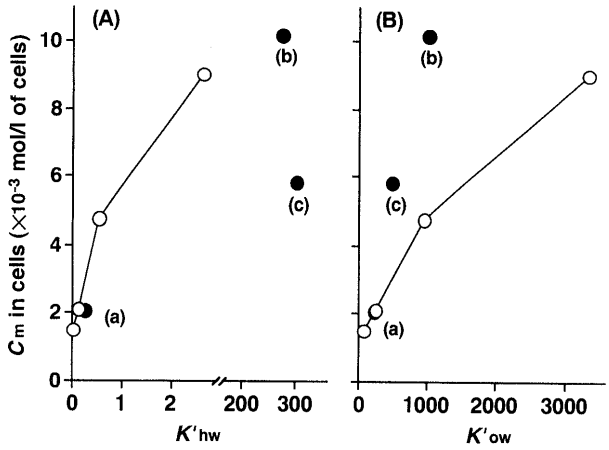

Fig. 4. Relationship between the C_m in *E. coli* Cells and (A) K'_{hw} and (B) K'_{ow} of Parabens and Ethyl Benzoate Derivatives

Solid line: parabens. Closed circle: (a), ethyl *m*-hydroxybenzoate; (b), ethyl *o*-hydroxybenzoate; (c), ethyl benzoate. C_m is the mean of three measurements.

that the uptake of the drugs into *E. coli* cells dose not depend solely on hydrophobic interactions. The C_m of both drugs was greater than that anticipated from the C_m *vs.* K'_{ow} curve obtained from parabens. This indicates the contribution of their greater hydrophobicity to the uptake into the cells. Consequently, the uptake of ethyl benzoate derivatives into bacterial cells, as well as that of parabens, may thus be concluded to be due not only to hydrophobic interactions between the alkyl or phenyl group and hydrophobic constituents in the cells but also to hydrogen bonding between the phenolic hydroxyl group or carbonyl oxygen atom and hydrophilic constituents.

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