

Determination of Structure-Toxicity Relationship of Amphiprotic Compounds by Means of the Inhibition of the Dehydrogenase Activity of *Pseudomonas putida*

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Aliphatic and aromatic alcohols are amphiprotic compounds which have both polar and nonpolar parts in their structure. These compounds were studied with respect to the nonreactive toxic effects on the microorganism *Pseudomonas putida*. The toxicity of these chemicals to aerobic bacterium *P. putida* was measured in terms of inhibition of dehydrogenase activity. The test results, expressed as concentration of chemicals 50% effective in inhibition (IC_{50}), were correlated with their physicochemical properties such as aqueous solubility (S) and octanol-water partition coefficient (P).

Introduction

Water pollution has become a major global environmental problem. Surface waters are heavily polluted with organic compounds persistent to biodegradation at an increasing rate every year. Most of the organic compounds can be degraded under aerobic conditions by different bacteria but some of them are persistent to aerobic degradation¹. Therefore, toxicity tests of chemicals using bacterial play an important role in their effects on the aquatic ecosystem. Since the bacteria have the shortest response time among the aquatic organisms to changes in their environment, they could be sensitive indicators of the hydrophobicity of organic compounds^{15,16}. In this respect, it has been used to predict the extent of bioaccumulation of organic pollutants in biological systems. Hansch *et al*¹⁴ first proposed a linear relationship between aqueous solubility and the octanol-water coefficient. Later, Chiou *et al*¹⁷ developed this relationship more quantitatively for environmental chemicals and subsequent correlations have been proposed by many researchers^{18–27}.

The objectives of the present study were to assess alcohol toxicity to bacteria since this group is an important component of the ecosystem, to use the bacteria as early indicators of environmental problems and to establish structure-activity relationships.

Experimental

Dehydrogenases are involved in vital anabolic and catabolic reactions and seem ideal for use in such toxicity studies²⁸. In this toxicity method, resazurin acts as an electron acceptor²⁹. Aliphatic and aromatic

alcohols act as nonspecific toxicants which have inhibitory effects on bacterial cells corresponding to chemical concentration. Toxicity measurements are based on the quantitative reduction of resazurin to resorufin by microbial dehydrogenase and is measured spectrophotometrically. Chemical reducing substances (aliphatic and aromatic alcohols) in water inhibit microbial dehydrogenase. This inhibition causes decreased reduction of resazurin which is related with the toxicity of these chemicals. The reduction of resazurin to resorufin by microbial dehydrogenase is shown in Figure 1.

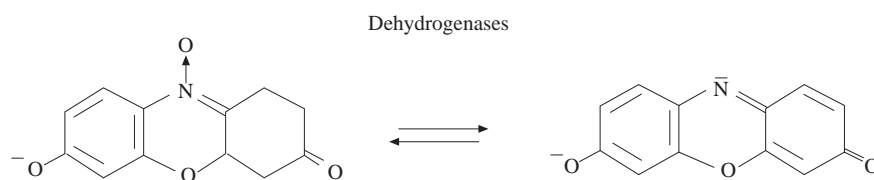


Figure 1. The reduction of resazurin to resorufin by dehydrogenase of *P. putida*.

Materials

Aliphatic alcohols with C₁ C₈ carbon chains, aromatic alcohols including substituted phenols, benzyl alcohol and 2-naphthol were used (Table 1). All compounds were purchased from E. Merck and Sigma Chem. Co and they were analytic reagent grade.

Test organism

P. putida is a soil and water organism isolated from putrid materials. *P. putida* strains will grow at 37° C. It is aerobic and facultative²⁸.

The lyophilized DSM-50026 strain of *P. putida* was purchased from the German Collection of Microorganisms and Cell Cultures (DSM-GmbH, Braunschweig, Germany). The bacteria were grown on Standard 1 nutrient agar^{28,29} (for approximately 18h) at 26° C. Prior to the experiments, the culture was transferred to a flask containing fresh Standard 1 nutrient broth (E. Merck). After overnight growth (16±2h) on a shaker at 21° C, 0.1 mL of the culture was transferred to another flask containing fresh medium. This transferring process was repeated until an active culture was established¹⁸. The cell concentration was adjusted to 0.55±0.05 O.D_{436 nm}.

Determination of toxicities

The experiments were carried out in standard 2x15 cm test tubes at 37° C in a water bath, adding 0.5 mL of culture to the tubes containing samples and reagent control which were prepared by the following method.

- A: Reagent control : 5 mL distilled water+0.5 mL imidazol buffer + 1 mL resazurin solution
- B: Blank sample : 5.5 mL borate buffer +1 mL resazuring solution
- C: Sample : mL solution of alcohol +0.5 mL imidazol buffer +1 mL resazurin solution.

Resazurin solution was prepared at 75 ppm concentration. The pH of the buffer solution of imidazol (0.1 M) was adjusted to 6.3 with 1.0 M hydrochloric acid. The pH of the borat buffer solution (0.1 M) was adjusted to 11.0 with 1.0 M sodium hydroxide.

Table 1. Relative toxicities (IC₅₀), hydrophobicity (log P) and solubility (log S) of amphiprotic compounds (Values are the mean of 5 experiments)

Comp. no	Compound	log P	log S (mol/L)	log IC ₅₀ (mg/L)
1	Methanol	-0.77	1.55	5.25
2	Ethanol	-0.31	1.23	5.36
3	1-Propanol	0.25	0.56	4.88
4	2-Propanol	0.05	1.60	5.00
5	1,2-Propanediol	-1.54	inf.	5.44
6	1,2,3-Propanetriol	-3.07	inf.	5.88
7	1-Butanol	0.88	0.08	4.35
8	2-Butanol	0.61	0.23	4.57
9	2-Methyl-1-propanol	0.83	0.11	4.49
10	2-Methyl-2-propanol	0.37	inf.	4.60
11	1-Pentanol	1.56	-0.61	3.59
12	1-Hexanol	2.03	-1.23	3.34
13	Cyclohexanol	1.23	0.38	4.05
14	1-Heptanol	2.41	-1.98	2.72
15	1-Octanol	2.97	-2.37	2.68
16	Phenol	1.46	-0.02	3.80
17	2-Methylphenol	1.95	-0.73	3.39
18	3-Methylphenol	1.96	-0.73	2.36
19	4-Methylphenol	1.94	-0.73	3.21
20	2-Aminophenol	0.62	-0.81	3.44
21	4-Aminophenol	1.01	-1.23	2.60
22	3-Nitrophenol	2.00	-1.01	3.05
23	4-Nitrophenol	1.91	-0.94	3.08
24	2,4-Dinitrophenol	1.51	-2.96(12.5°C)	2.01
25	1,2-Dihydroxybenzene	0.88	0.60	3.79
26	1,3-Dihydroxybenzene	0.80	0.96	4.24
27	1,4-Dihydroxybenzene	0.59	-0.19	3.78
28	1,2,3-Trihydroxybenzene	–	0.54(13°C)	4.59
29	2-Chlorophenol	2.15	-0.65	3.38
30	3-Chlorophenol	2.50	-0.69	2.18
31	4-Chlorophenol	2.39	-0.68	2.90
32	4-Ethylphenol	2.58	0.38	2.92
33	Benzyl alcohol	1.10	-0.43	3.39
34	2-Naphthol	2.88	-2.28	2.73

After an incubation time of 75 min, 1 mL borate buffer and 10 mL *n*-amyl alcohol were rapidly added to each tube to stop the reaction. The upper alcohol layer was transferred to a test tube containing 2 g of sodium bicarbonate. The tubes were centrifuged at 4000 rpm for 5 min and the absorbance of each sample was measured with a spectrophotometer at 615 nm.

Toxicity to the *P. putida*, expressed as percent inhibition (I %), of the sample was calculated using the following equation:

$$I\% = \frac{(B - A) - (B - C)}{(B - A)} \times 100 \quad (1)$$

Where A: Absorbance of the reagent
 B: Absorbance of the blank
 C: Absorbance of the sample

Results and Discussion

The IC_{50} values of the toxicants were calculated by interpolation of a plot of percent inhibition versus concentration. The observed IC_{50} of each chemical is given in Table 1.

The octanol-water partition coefficients (P) and water solubilities (S) of the organics were used as reported in the literature^{3,4,15-27}. In order to obtain a simple linear correlation between toxicity (IC_{50}) and P, IC_{50} and S, the results were represented on a logarithmic scale. The values for log S in Table 1 were selected at 20-25°C.

The regression analysis of log IC_{50} versus log P and log S were assessed with the Cricket Graph program on a Macintosh computer.

The relationship between IC_{50} and log P for 13 different aliphatic alcohols were expressed by a simple equation (eq.2) which was derived from experimental data.

$$\log IC_{50} = 4.98 - 0.18 \log P \quad (2)$$

$n = 13$, $r^2 = 0.98$ (n is the number of toxicants analyzed, r^2 is the correlation coefficient).

This data and the relationship are shown in Figure 2.

Equation 2 was a relationship between log IC_{50} and log P for aliphatic alcohols and with their isomers except compounds 5 and 6 which are in Table 1. Equation 2 has a high correlation coefficient ($r^2 = 0.98$), which means that toxicities of aliphatic alcohols to *P. putida* are simply dependent on the hydrophobicity (log P).

Similar simple equations were also expressed between log IC_{50} and log S (eq. 3) for aliphatic alcohols. This equation was obtained from Figure 3.

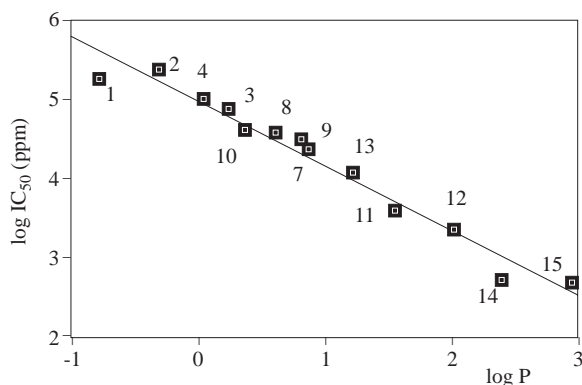


Figure 2. Relationship between log IC_{50} and log P for C_1 - C_8 normal and for C_3 - C_4 branched alcohols

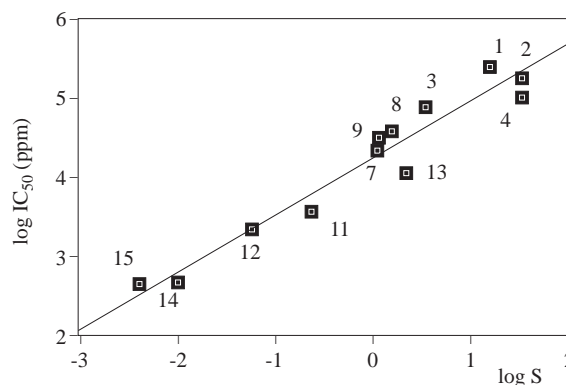


Figure 3. Relationship between log IC_{50} and log S for C_1 - C_8 normal and for C_3 - C_4 branched alcohols

$$\log IC_{50} = 4.22 + 0.69 \log S \quad (n = 12, r^2 = 0.98). \quad (3)$$

IC_{50} collineated with $\log S$ for aliphatic alcohols with high correlation coefficients ($r^2 = 0.98$). Data for compounds 5, 6 and 10 were not included because these compounds are infinitely soluble in water. Inhibition effects of aliphatic alcohols on dehydrogenase activity of *P. putida* increased linearly with the increased number of carbon atoms (greater than 4C) in the hydrocarbon chain. Branching at the aliphatic chain was shown to reduce the toxicity corresponding to higher aqueous solubility and lower octanol-water partition coefficient as seen when *sec*-butanol and *tert*-butanol were compared with *n*-butanol (Table 1). Decreased hydrophobicity due to multiple -OH groups resulted in lower toxicity, as seen in the results for 1,2-propanediol and 1,2,3-propanetriol compared with 1-propanol and 2-propanol. The test results also showed that alicyclic alcohol exhibited a lower inhibiting effect on enzyme activity than open-chain alcohol compared with cyclohexanol depending on lower $\log P$ and higher $\log S$ (Table 1).

Poor linear correlations (eqs. 4 and 5) were obtained between $\log IC_{50}$ and $\log P$ and $\log IC_{50}$ and $\log S$ for aromatic alcohols (Figures 4 and 5), whereas the correlations were quite high for aliphatic alcohols.

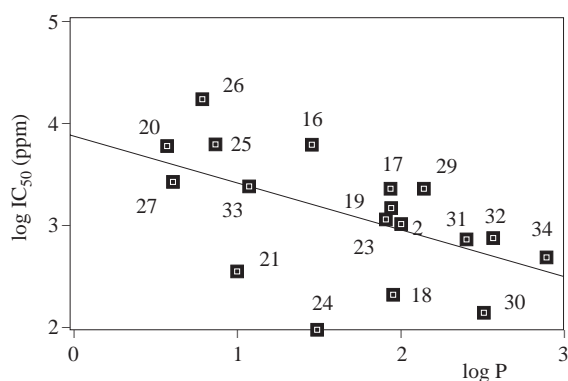


Figure 4. Relationship between $\log IC_{50}$ and $\log P$ for aromatic alcohols.

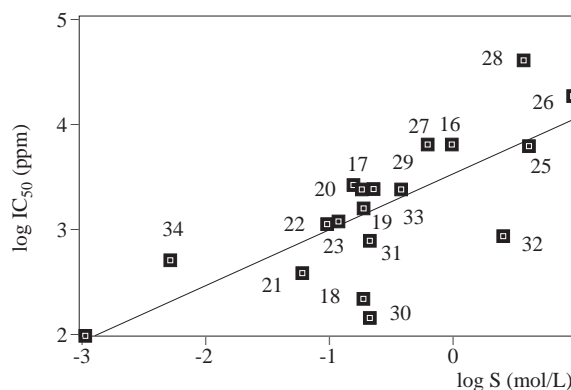


Figure 5. Relationship between $\log IC_{50}$ and $\log P$ for aromatic alcohols.

$$\log IC_{50} = 3.89 - 0.46 \log P \quad (n = 18, r^2 = 0.54). \quad (4)$$

$$\log IC_{50} = 4.22 + 0.69 \log S \quad (n = 20, r^2 = 0.75). \quad (5)$$

However the plots in Figure 4 and Figure 5 show some groups of data points. There is a correlation between toxicity and hydrophobicity. In Figure 4, -OH and -NH₂ substituted phenols and benzyl alcohol simply have lower $\log P$ values and thus lower toxicity on average, but the scatter in their toxicity overlaps with the other chemicals and does not clearly segregate from the other chemicals.

Poor correlation between $\log IC_{50}$ and $\log P$ and $\log IC_{50}$ and $\log S$ were observed for aromatic alcohols. One possibility is that some of these chemical are not nonreactive nonspecific toxicants. Solubility data for compounds 24 and 28 were determined at 12.5°C and 13°C but for the others at 20-25°C.

Aromatic alcohols were included in the regression analysis of aliphatic alcohols and simple correlations were obtained between $\log IC_{50}$ and $\log P$ in Figure 6 and $\log IC_{50}$ and $\log S$ in Figure 7 with correlation coefficients $r^2 = 0.83$ and $r^2 = 0.85$, respectively. The relation between $\log IC_{50}$ and $\log P$, $\log S$ for studied amphiprotic compounds were obtained as in eqs. 6 and 7.

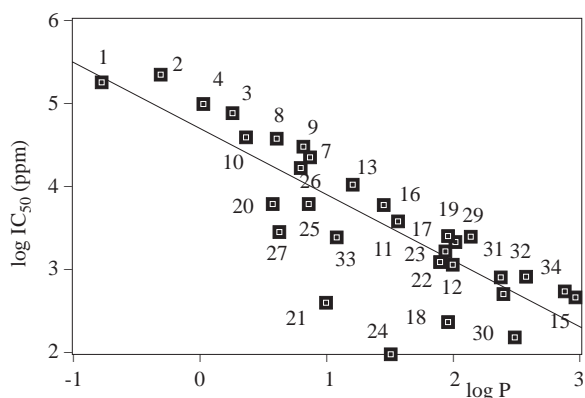


Figure 6. Relationship between $\log IC_{50}$ and $\log P$ for aliphatic and aromatic alcohols

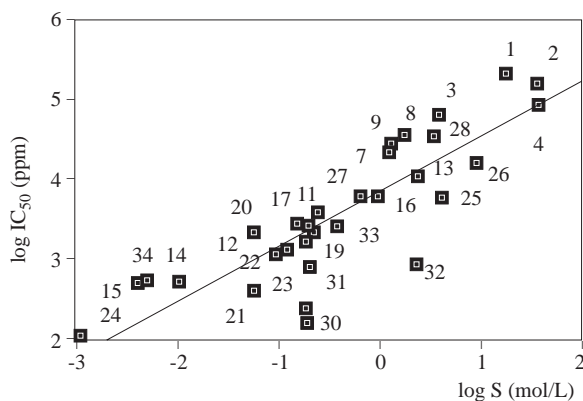


Figure 7. Relationship between $\log IC_{50}$ and $\log S$ for aliphatic and aromatic alcohols

$$\log IC_{50} = 4.67 - 0.80 \log P \quad (n = 31, r^2 = 0.83). \quad (6)$$

$$\log IC_{50} = 3.85 + 0.69 \log S \quad (n = 12, r^2 = 0.85). \quad (7)$$

Similar simple equations have also been used for the toxicity of alkanols, substituted benzenes, and alkanes to the guppy³⁰, rainbow trout³² and fathead minnow³². Bacterial data can be used in correlations. A correlation between *Nitrobacter* and *P. putida* has correlation coefficient of 0.78 (Figure 8).

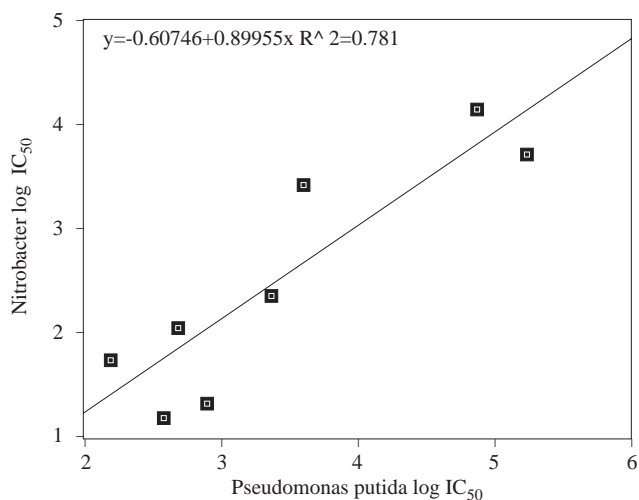


Figure 8. Correlation between toxicity to *Nitrobacter* and *P. putida*

The simple equations that we obtained may be used to predict the potential toxicity of any aliphatic or aromatic alcohol with known aqueous solubility and octanol-water perturbation².

The toxicity of organic chemicals to aerobic heterotrophs *Nitrosomonas*, *Methanogenes* and marine bacterium *Photobacterium phosphoreum* has been studied by Blum and Speece³. Tange *et al.*⁴ researched the toxic effects of selected phenols, benzenes and aliphatics to *Nitrobacter*. Ewald⁵ and Liu^{6,7} used a modified test method for the determination of the activity of dehydrogenases in an activated sludge and sediment. Since *Pseudomonas putida* is the most efficient bacterium of activated sludge, it is environmentally significant

and important in wastewater treatment processes^{8,9,10}. Chemical toxicity to *P. putida* was determined in terms of inhibition of cell multiplication (growth inhibition test)¹¹ and oxygen uptake¹².

Aliphatic and aromatic alcohols represent an important class of compounds from an industrial standpoint. Many alcohols are manufactured and sold by the ton throughout the world. Many of these are important in the manufacture of surfactants, plasticizers, cosmetics, lubricants, evaporation suppressors, and numerous other products. The hydroxyl group is moderately reactive chemically; thus, the alcohols are involved either as reactants or products in a wide variety of chemical reactions which include esterification and hydrolysis, dehydration and hydration, dehydrogenation and hydrogenation, and oxidation and reduction¹³.

The toxic mechanism of alcohols and substituted phenolics that are nonreactive toxicants, according to Blum and Speece³ are dependent on the quantity of toxicant acting upon the cell. The potency of a nonspecific (or nonreactive) toxicant, at equilibrium in an aquatic system, is controlled by the partitioning of the toxicant between the water and the hydrophobic components of the biophase. The selection of a suitable solvent pair (water and an apolar liquid) to use as a model system for the aqueous and fatty phases of biological system is an important problem. Hansch *et al*¹⁴ have used an octanol-water pair to serve as a model for the aqueous and lipid phases for living tissue. The partition coefficient (P) is a useful physicochemical property for characterizing the lipophilicity or partition coefficient.

All the predicted values (Table 2) from Equations 6 and 7 within the confidence interval of the equations.

Table 2. Predicted IC₅₀ of some aliphatic and aromatic alcohols in validation of Equations 6 and 7

Toxicant	log S (mol/L)	log P	Predicted IC ₅₀ from eq.6	Predicted IC ₅₀ from eq. 7
2-Hexanol	-0.867 ^a	1.61 ^a	3.38	3.25
2-Hexanol	-0.795 ^a	1.61 ^a	3.38	3.30
2-Pentanol	-0.279 ^a	1.14 ^a	3.76	3.65
3-Pentanol	-0.211 ^a	1.14 ^a	3.76	3.70
2,4-Dimethyl phenol	-1.19 ^b	2.42 ^b	2.73	3.02
2,4-Dichloro phenol	1.44E-3 ^c	1.67 ^d	3.33	3.85
2,4,6-Trichloro phenol	0.60E-3 ^c	2.08 ^d	3.00	3.85

^a Values were taken from Reference 14

^b Values were taken from Reference 18

^c Values were taken from Reference 27

^d Values were taken from Reference 4

This method has advantages over the other test methods such as easy cultivation and shorter time requirement (appr. 1 h). Thus the resazurin test can readily be used in structure-toxicity studies where rigid control and accuracy are needed to obtain comparative data. Obviously there is still room for further improvement of this test.

Acknowledgement

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