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Koshland's model as a method for the analysis of enzymatic deracemization reactions

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Koshland's four-point location model is applied to consider some enzymatic reactions of deracemization. The sequential model is a theory describing the cooperativity of protein subunits. It postulates that the conformation of the protein changes with each binding of a ligand, thus sequentially changing its affinity to ligands at adjacent binding sites. When the substrate binds to the active site of one subunit of the enzyme, the remaining subunits are activated. The possibility of the alternative binding of both substrates and products of the enzymatic reaction can be assessed on the basis of the known data on the structure of all four substituents at the chiral atom and their correspondence to the ligand specificity of the corresponding subsets of the enzyme. The made theoretical conclusions were tested by the example of the enzymatic deracemization of some hydroxyphosphonic acids. The replacement of ethoxyl groups at the phosphorus atom by isopropoxy groups and an increase in the volume of the substituent led to a significant increase in the enantiomeric excess of the hydrolysis product of hydroxyphosphonate. Hence, the conclusion is drawn that the key criterion for the efficient or inefficient passage of the reaction is the ratio of the sizes and the degree of hydrophobicity of the corresponding substituents at the asymmetric reaction center.

Keywords: *Koshland's model, lipases, enzymes, kinetic resolution, Candida Antarctica lipase.*

The production of chirally pure substances occupies a special place among the priority areas of modern biotechnology, since the enantiomers of the same substance are qualitatively different in biological effects. The classical approach to ensuring this kind of purity is to use enzymes whose stereoselectivity provides a reaction with only one of the enantiomers [1]. Achieving the maximum yield of a target product is an essential condition for the optimization of any technological process. In this case, however, complications are possible due to the possibility of the alternative binding of a reaction product, which becomes, in this case, a competitive inhibitor of the active center of the enzyme. An increase in the concentration of such a product inevitably leads to a decrease in the reaction rate and, as a consequence, to a blockage of the process. In this work, we consider the case of this kind of blocking by the example of enzymatic acylation of optically active alcohols. Consideration of this case is of interest not so much to explain the low efficien-

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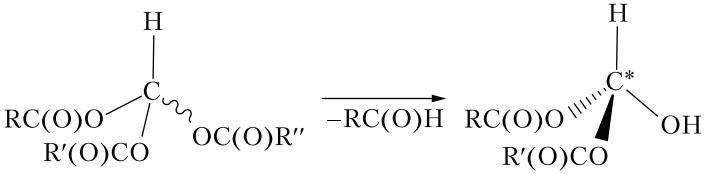
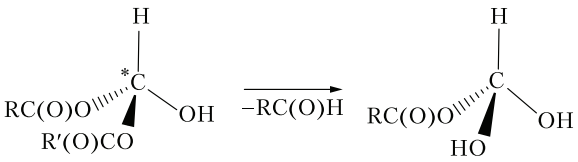
cy of the enzymatic synthesis, but to prevent failures by choosing an appropriate strategy for stereospecific synthesis.

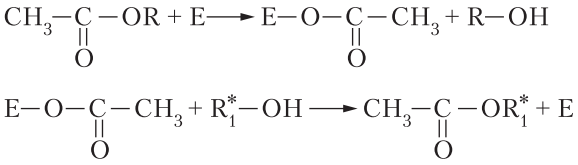
Enzymatic cleavage of neutral fats to monoacylglyceride and fatty acids occurs in two stages and is catalyzed by lipases. When the first fatty acid residue is cleaved off, an optically active diacylglyceride is formed (hereinafter — an optically active compound):



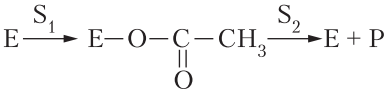
The cleavage of the second residue leads to the disappearance chirality:



Like all enzymes, lipases are capable of catalyzing both direct and reverse reactions, i.e., under certain conditions, facilitating the transfer of the acyl group to the alcohol group of a compound suitable for stereospecificity. This ability has found application in the stereoselective acylation of optically active alcohols. The process takes place in two steps — the formation of the acyl enzyme and the transfer of the acyl group to one of the enantiomers of the optically active alcohol:

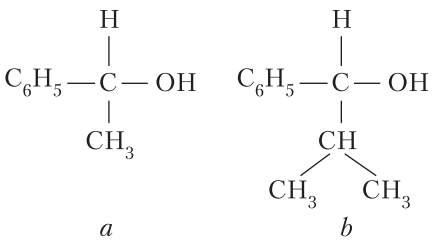


In other words, a sequential two-substrate reaction occurs, by leading to the stereoselective acylation of one of the enantiomers introduced into the reaction:



In our case, the enzymatic component of the process is a preparation of lipase immobilized on an insoluble carrier. The use of acyl-propenyl as the first substrate makes the first step practically irreversible, since the propenol released during the reaction is immediately isomerized to acetone. The acyl enzyme formed during the first step transfers the acyl group to only one of the enantiomers of the optically active alcohol, thereby forming a chiral pure acylated derivative. In the case of phenyl-methyl-carbinol (Fig. 1, *a*), the reaction was almost quantitative, ensuring the complete acylation of one enantiomer without affecting the other. In the case of phenyl-isopropyl-carbinol (see Fig. 1, *b*), only 12 % of the enantiomer is acylated, after which the accumulation of the product and, hence, the course of the reaction cease.

This difference can be explained on the basis of the Koshland four-point location model [2]. When the substrate binds to the active site of one subunit of the enzyme, the remaining subunits



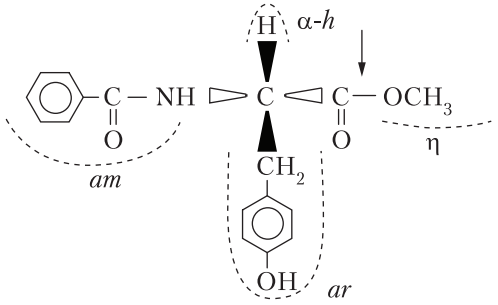


Fig. 1. Phenyl-methyl-carbinol (*a*) and phenyl-isopropyl-carbinol (*b*)

Fig. 2. Placement of methyl ester of N-benzoyl-L-tyrosine in the active center of α-chymotrypsin [3, 4]. (Hereinafter, the α-carbon bonds protruding from the plane are painted over, and those extending into the interior are light, which corresponds to the L-enantiomer of the amino acid)

are activated. Ligands can have non-cooperative, positive, or negative cooperative effects. An example of positive cooperativity is the binding of oxygen by hemoglobin. Negative cooperativity means the opposite: at the time moment of the binding of a ligand to the protein, the affinity of the protein to other ligands decreases. An example is the interaction between glyceraldehyde-3-phosphate and the enzyme glyceraldehyde-3-phosphate dehydrogenase. Thus, the strength of the interaction of atoms or molecules increases as changes in the system increase, making them collectively consistent. According to this model, in the process of "cooperative transitions", the optically active substrate interacts with the active center of the enzyme through all four substituents of the chiral atom. Examples of the interaction of chymotrypsin-trypsin-like proteinases with a number of low-molecular-weight substrates are most indicative in this respect. The orientation of the hydrolyzable bond in the zone of action of the catalytic center is set by the placement of all four substituents of the chiral atom in the corresponding loci of the enzyme (Fig. 2).

In this case, the specificity of the action of the enzyme is determined by the properties of the *ar*-site, the binding site, and the hydrophobic pocket of the enzyme. In the case of chymotrypsin, it is complementary to hydrophobic amino acid residues. The *am*-site interacting with the acylamide group has little effect on the binding strength of the substrate, but contributes to the correct orientation of the cleavable bond into the zone of action of the hydrolytic center. The α-*h* zone is small, and the placement of any residue larger than a hydrogen atom in it sharply decreases the reactivity of the substrate or inhibitor [5]. On the contrary, placing a bulky hydrophobic substituent in the binding zone of the "leaving group" η is very advantageous, since it provides the correct orientation of the substrate with the direction of the hydrolyzable bond in the zone of action of the catalytic center. Due to this four-point interaction, proteolytic enzymes cleave amide and ester bonds formed by carboxyl groups of only L-amino acids. In Fig. 2, we show the active site occupied by α-chymotrypsin of a popular low-molecular-weight substrate — methyl ester of N-benzoyl-L-tyrosine. The hydrophobic tyrosine radical is located in the *ar*-site, the acylamide group — in the *am*-site, the α-hydrogen atom — in the α-*h*-site, while the cleavable bond is oriented toward the zone of action of the hydrolytic center. The bulky N-benzoyl group better ensures the proper orientation of the hydrolyzable bond and, hence, the rate of hydrolysis, than the N-acetyl group, which can be seen from a comparison of the kinetic parameters of the chymotrytic hydrolysis of N-benzoyl and N-acetyl-L-tyrosyl ethers [3] ...

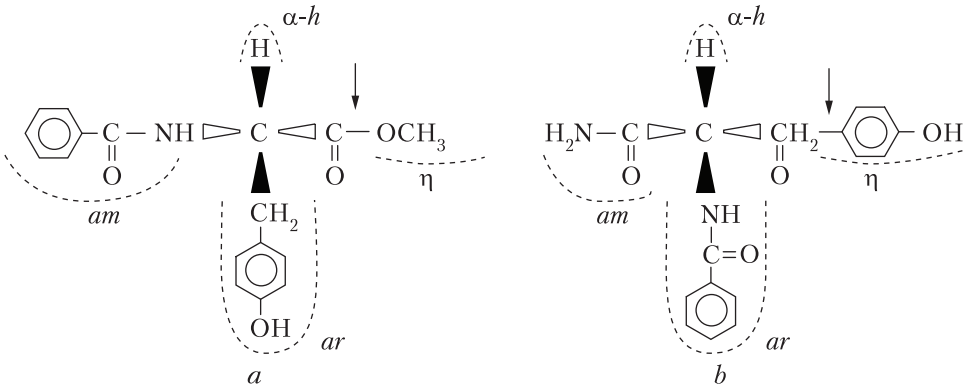


Fig. 3. Variants of the N-benzoyl-L-tyrosyl-amide placement in the active center of α-chymotrypsin: *a* — productive, *b* — unproductive

On the other hand, for the same reasons, when these enzymes interact with a number of low molecular weight compounds, the so-called unproductive binding is observed, namely, the formation of an enzyme-substrate adduct that does not lead to the formation of a reaction product. The unproductive binding of a substrate by an enzyme is similar to the competitive inhibition, with the only difference in that the substrate itself acts as a competitive inhibitor:

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$
$$\Big\downarrow\uparrow K_s'$$
$$ES'$$

The equation for the rate of formation of the reaction product in this case is described by the equation [6, p. 150-152]:

$$v = \frac{k_2[E]_0[S]_0}{K_s + (1 + K_s / K_s')[S]_0}.$$

The degree of influence of the formation of the unproductive complexation on the reaction rate is determined by the ratio of the constants K_s and K_s'. A typical manifestation of the unproductive binding may be invulnerability against the chymotryptic hydrolysis of N-benzoyl-L-tyrosyl-amide [7], while N-acetyl-L-tyrosyl-amide is hydrolyzed quite efficiently [8]. N-benzoyl-L-tyrosyl-glycine is also not hydrolyzed by chymotrypsin [7]. This can be explained by the efficiency of the non-productive binding of the substrate by the enzyme (Fig. 3).

The possibility of binding the benzoylamide group by the *ar*-site of chymotrypsin is confirmed by the efficient hydrolysis of hippuric acid derivatives (N-benzoyl-glycine) [9, p. 198-200]. On the other hand, trypsin, which differs from chymotrypsin in the specificity of the *ar*-site, quite effectively hydrolyzes N-benzoyl-L-lysyl-amide and N-benzoyl-L-arginyl-amide [10]. N-benzoyl-L-tyrosyl is no less indicative -glycyl-amide, which is efficiently hydrolyzed by α-chymotrypsin, while its D-isomer not only does not hydrolyze itself, but also completely inhibits the hydrolysis of the L-isomer [7]. On the one hand, the "leaving group" of N-benzoyl-L-tyrosyl-glycyl-amide, which is more bulky compared to N-benzoyl-L-tyrosyl-amide (see Fig. 3, *a*),

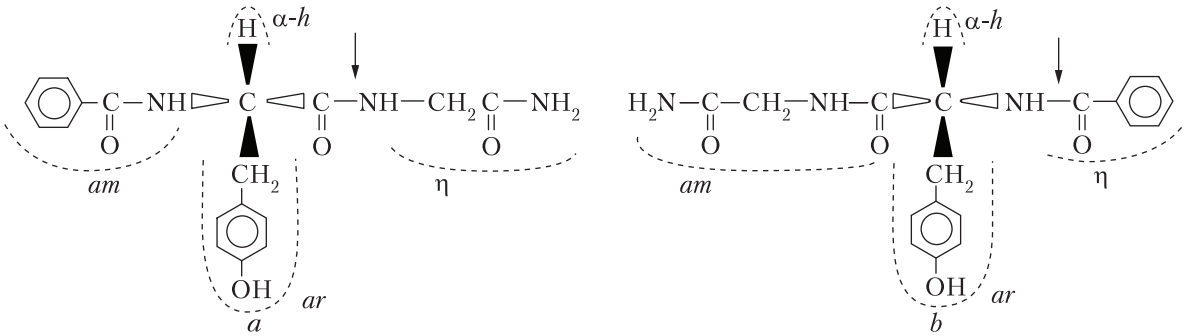


Fig. 4. Productive placement of N-benzoyl-L-tyrosyl-glycyl-amide (*a*) in the active site of α-chymotrypsin and the unproductive placement of N-benzoyl-D-tyrosyl glycyl-amide (*b*)

promotes the productive placement of the substrate in the active center of the enzyme (Fig. 4, *a*). The alternative placement of the D-enantiomer is efficient for the same reasons (see Fig. 4, *b*).

Placing a small amide residue (the case of N-benzoyl-L-tyrosyl-amide) or a free carboxyl group of glycine (the case of N-benzoyl-L-tyrosyl-glycine) in the binding zones of the "leaving group" destabilizes the system, by making the alternative unproductive binding beneficial. In all the cases considered, only the states corresponding to the steric specificity of the enzyme of all four enzyme subsets are realized. Placing only the α-hydrogen atom in the α-*h* zone turns out to be a critical condition.

The presented materials make it possible to explain the limitation of the transfer of acyl to the corresponding enantiomer of phenyl-isopropyl-carbinol and the complete acylation of the enantiomer of phenyl-methyl-carbinol. The resulting end products differ from each other only in the level of hydrophobicity of one of the substituents of the chiral atom, which, however, turns out to be a sufficient circumstance for an efficient alternative binding of the enzyme by the end product of the reaction. The resulting product becomes an efficient competitive inhibitor that, when a certain concentration is reached, completely blocks the reaction. In this case, two points deserve attention. First, the end product of the second reaction step inhibits the first step. In this case, the kinetic parameters of the second step have no particular effect on the dynamics of the process. The simplified reaction scheme takes the form, where S_1 and S_2 are substrates of the first and second reactions, and P* is the final reaction product, which has the ability to alternatively be bound by the active center of the enzyme:

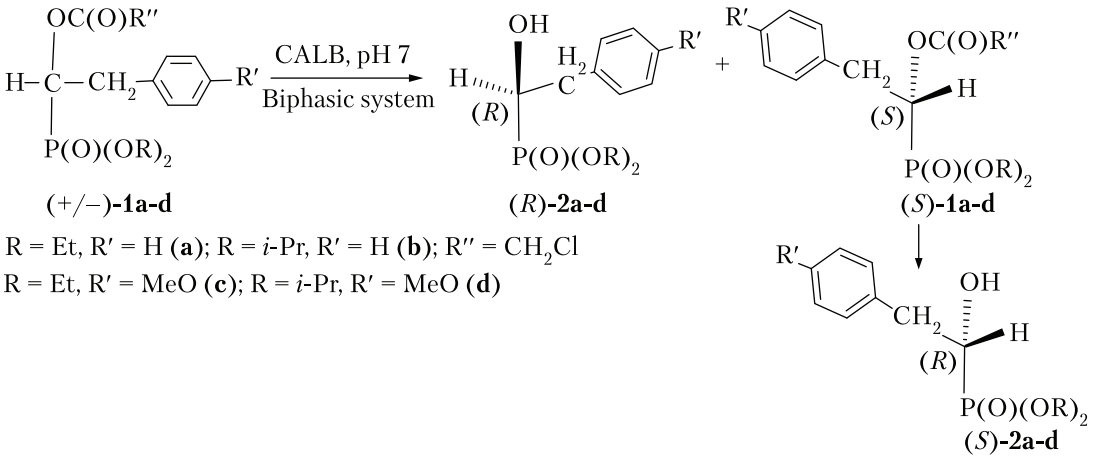
$$E \xrightarrow{S_1} AcOE \xrightarrow{S_2} E + P^*$$
$$\downarrow\uparrow P^*$$
$$EP^*$$

The possibility of blocking the formed acyl-enzyme by the product seems unlikely due to steric hindrances created by the acyl group bound to the enzyme. Second, during the reaction, the concentration of the final product increases, which is equivalent to an increase in the concentration of a competitive inhibitor. In our case, as $P^* \to 0.12\ S_2$, $v \to 0$.

It is obvious that increasing the concentration of S_1 will hardly be efficient for increasing the yield of the target product. The key criterion for the efficient or inefficient reaction is the ratio of the sizes and the degree of hydrophobicity of the corresponding substituents of the chiral

atom. Thus, in our case, the Hanlon hydrophobicity parameter (the logarithm of the distribution of the substance content in the *n*-octanol / water system) for benzene is 2.22, for propane, 2.35, and only 1.09 for methane. Therefore, the isopropyl and phenyl substituents are significantly superior to the methyl one both in terms of bulk and hydrophobicity. The same trend persists in the case of amides of benzoic, isobutyric, and acetic acids (0.74, –0.36, and –1.23, respectively). It is also worth noting that Koshland's requirement to consider the nature of all four substituents of a chiral atom with the exclusion of replacement of a hydrogen atom by a more bulky group is obligatory not only for the "retaining group," but also for the "leaving" one.

We used our theoretical conclusions for practical experimental separations of hydroxyphosphonic acids



R = Et, R′ = H (**a**); R = *i*-Pr, R′ = H (**b**); R″ = CH_2Cl
R = Et, R′ = MeO (**c**); R = *i*-Pr, R′ = MeO (**d**)

Deracemization of hydroxyphosphonic acid acetates was carried out by the biocatalytic hydrolysis in a two-phase system MTBE-buffer solution with constant pH 7 in the presence of CAL-B lipase applied to the polymer.

The hydrolysis reaction was carried out to approximately 50 % conversion of aetate to hydroxyphosphonate. The reaction was stopped by the filtration of the biocatalyst. The progress of the reaction was monitored by ^{31}P NMR. The reaction products, acetate and hydroxyphosphonate, were separated by column chromatography on silica gel. The enantiomeric purity of the products was determined by the derivatization with Mosher's acid. The Kazlauskas rule was used to determine the absolute stereochemistry of enantiomerically pure products. The Kazlauskas rule is an empirical model based on the postulate that the enantioselectivity is proportional to the difference in size between large (L) and middle (M) substituents in the substrate. According to the Kazlauskas rule, these substitutes are located in two different pockets of the active site of an enzyme, according to their size, which determines the absolute configuration of

Enzymatic deracemization of hydroxyphosphonates

Entry	R	R′	Yield **2**, %	*ee* **2**, %	Yield **3**, %	*ee* **3**, %
1	Et	H	40	90	50	65
2	*i*-Pr	H	45	95	50	70
3	Et	MeO	40	20	40	20
4	*i*-Pr	MeO	45	80	55	80

the products in the enzymatic reaction. Using this empirical rule, it was found that the biocatalytic acylation produces (*R*)-esters, and (*S*)-halogenindanol remains unreacted.

The results shown in Table indicate that the replacement of ethyl R groups by isopropyl groups and an increase in the volume of the substituent led to a significant increase in the enantiomeric excess of the hydroxyphosphonate hydrolysis product, although the reaction time increased significantly (from 20 h to 65 h). In accordance with the conclusions presented above, the key criterion for the efficient or inefficient passage of the reaction is the ratio between the sizes and the degree of hydrophobicity of the corresponding substituents of the chiral atom.

From the material presented, it follows that the possibility of the alternative binding of both the substrate and the product of the enzymatic reaction can be estimated on the basis of the known data on the structure of all four substituents of the chiral atom and their correspondence to the ligand specificity of the corresponding enzyme subsets. Such an assessment is necessary in the planning of biotechnological processes and allows one to avoid the unproductive loss of expensive reagents and working time.

Experiment. ^{1}H NMR and ^{13}C NMR spectra were recorded in a $CDCl_3$ solvent on a 500 MHz spectrometer at ambient temperature. Chemical shifts (δ) are shown in ppm in relation to TMS as an internal standard. Signal multiplicity is shown as s — singlet; d — doublet; dd — doublet of a doublet; etc., dt — triplet of doublets; t — triplet; m — multiplet; br. s — wide singlet. The spin-spin coupling constants *J* are indicated in Hz. All reagents and solvents were used without special purification, unless otherwise indicated. Column chromatography was performed on silica gel 60 (70-230 mesh) using the indicated eluents. Optical rotations were measured on a Perkin-Elmer 241 polarimeter (D sodium line at 20 °C). Melting points were not corrected. All reactions were carried out in glassware dried on a fire or dried in a drying chamber with stirring on a magnetic stirrer. Lipase from *Antarctica Candida lipase B* (Novozim 435) was purchased from Sigma-Aldrich. The progress of the reactions was monitored by NMR. The purity of all compounds was checked, by using thin-layer chromatography and NMR measurements.

Enzymatic hydrolysis of dialkyl I-(acyloxyalkyl)phosphonates 1a-d.

Phosphonate **1** (1 mmol) was placed in a 25-ml flask, followed by the addition of organic solvents and sterile 0.05 M phosphate buffer (15 ml; prepared by dissolving 25 mmol KH_2PO_4 in 300 ml of distilled water with the addition of 1 N NaOH to bring pH to 7, followed by adding water to a final volume of 500 ml and then by autoclaving at 121 °C for 20 min). The mixture was vigorously stirred in a water bath at a constant temperature, and 0.5N hydrochloric acid was added, by using an autotitrator, to NaOH to adjust the pH to 7.0. When the enzyme was added, the pH was brought back to 7.0 and maintained by the automatic addition of base. When the appropriate amount of base was added, 1N HCl was added to bring the pH to 4.0. The mixture was filtered through celite, and the filtrate was extracted with ethyl acetate. The organic layers were combined, dried ($NaSO_4$), and concentrated. Unreacted ester **1** and hydroxyphosphonate **2** were separated by flash chromatography (see Table).

The unreacted ester **1** was dissolved in dry methanol (5 ml) and triethylamine (1 ml) and stirred at room temperature till the completion (TLC, about 24 h). The solution was concentrated, and the crude product was purified by flash chromatography to give α-hydroxyphosphonate **2**.

As a result, hydroxyphosphonates **1a-d** of (*S*)- and (*R*)-absolute configuration are obtained and are described below.

Diethyl 1-hydroxy-2-phenylethylphosphonate [(S)-2a]. m.p. 60 °C, $[\alpha]_D^{20}$ = +20.5 (C=1, $CHCl_3$) [11].

^{1}H NMR ($CDCl_3$): δ 1.31, 1.33 dt (6H, J = 6.9, CH_3), 3.03 m (2H, PhC$\underline{H}_2$), 3.25 br (lH, OH), 4.10 (1H, m, CHP), 4.16 (4H, m, OCH_2), 7.20 (5H, m, C_6H_5).

^{13}C NMR ($CDCl_3$) d, 16.8 (d, J_{PC} = 3.5 Hz); 16.9 (d, J_{PC} = 4 Hz), 37.9, 62.8 (d, J_{PC} = 7 Hz), 62.9 (d, J_{PC} = 7 Hz), 69.6 (d, J_{PC} = 160 Hz), 126.8, 128.9, 130.3, 138.2.

^{31}P NMR ($CDCl_3$): 24.1.

Diethyl 1-hydroxy-2-phenylethylphosphonate [(R)-2a]. $[\alpha]_D^{20}$ = –20.1 (C=1, $CHCl_3$).

^{1}H NMR ($CDCl_3$): δ 1.31, 1.33 d t (6H, J = 6.9, CH_3), 3.03 m (2H, PhC$\underline{H}_2$), 3.25 br (1H, OH), 4.10 (1H, m, CHP), 4.16 (4H, m, OCH_2), 7.20 (5H, m, C_6H_5) [11].

^{31}P NMR ($CDCl_3$): 24.1

Diisopropyl 1-hydroxy-2-phenylethylphosphonate [(S)-2b]. The solvent was removed in vacuo, and the residue was diluted with water (20 ml). The product was extracted with methylene chloride (3 × 20 ml). The extracts were dried (Na_2SO_4) and concentrated. The residue was resolved by flash chromatography (R_f = 0.33; methylene chloride/ ethyl acetate = 5 : 3) to afford the α -hydroxyphosphonate (*R*)-**2b** as colorless oil.

$[\alpha]_D^{20}$ = +21.1 (C=1, $CHCl_3$).

^{1}H NMR: d 1.31, 1.33, dd (6H, J = 6.4, CH_3), 2.65 br (1H, OH), 3.00 m (2H, $PhCH_2$), 4.02 dt (lH, CHP), 4.77 m (2H, OCH), 7.26 m (5H, C_6H_5).

^{31}P NMR ($CDCl_3$): 26.5.

Diisopropyl 1-hydroxy-2-phenylethylphosphonate [(R)-2b]. $[\alpha]_D^{20}$ = –21.5 (C=1, $CHCl_3$).

^{1}H NMR: d 1.31, 1.33, dd (6H, J = 6.4, CH_3), 2.65 br (1H, OH), 3.00 m (2H, $PhCH_2$), 4.02 dt (1H, CHP), 4.77 m (2H, OCH), 7.26 m (5H, C_6H_5).

^{31}P NMR ($CDCl_3$): 26.5.

Diethyl 1-hydroxy-2-(4-methoxyphenyl)ethylphosphonate [(S)-2c]. Purification by flash chromatography (methylene chloride/ ethyl acetate = 5 : 3) afforded the α-hydroxyphosphonate (*R*)-**2d** (49 %).

$[\alpha]_D^{20}$ = +18.0 (C=1, $CHCl_3$).

^{1}H NMR: δ 1.33, 1.34 dt (6H, J = 7.4, CH_3), 2.75 br.s (1H, OH), 2.99 m (2H, $PhCH_2$), 3.79 s (3H, OCH_3), 4.05 m (1H, CHP), 4.17 m (4H, OCH_2); 7.02 m (4H, C_6H_4).

^{13}C NMR ($CDC1_3$) d, 16.4 (d, J_{PC} = 3.5 Hz), 16.6 (d, J_{PC} = 4 Hz), 38.2, 62.5 (d, J_{PC} = 7 Hz), 62.2 (d, J_{PC} = 7 Hz), 68.3 (d, J_{PC} = 160.0 Hz), 126.1, 128.0, 129.9, 138.2.

^{31}P NMR ($CDCl_3$): 23.90.

Diethyl 1-hydroxy-2-(4-methoxyphenyl)ethylphosphonate [(R)-2c]. $[\alpha]_D^{20}$ = –17.2 (C=1, $CHCl_3$).

^{1}H NMR: δ 1.33, 1.34 dt (6H, J = 7.4, CH_3), 2.75 br.s (1H, OH), 2.99 m (2H, $PhCH_2$). 3.79 s (3H, OCH_3), 4.05 m (1H, CHP), 4.17 m (4H, OCH_2); 7.02 m (4H, C_6H_4).

^{31}P NMR ($CDCl_3$): 23.90.

Diisopropyl 1-hydroxy-2-(4-methoxyphenyl)ethylphosphonate [(S)-2d]. Purification by flash chromatography (methylene chloride/ethyl acetate = 5 : 3) afforded the α-hydroxyphosphonate (*R*)-**2d** (49 %). $[\alpha]_D^{20}$ = +20.1 (C=1, $CHCl_3$).

^{1}H NMR: δ 1.31, 1.33, dd (6H, J = 6.4, CH_3), 2.73 br (1H, OH), 2.96 m (2H, $PhCH_2$), 3.79 s (3H, OMe), 3.96 m (1H, CHP), 4.77 m (2H, OCH), 7.02 m (4H, C_6H_4).

^{31}P NMR ($CDCl_3$): 26,8.

Diisopropyl 1-hydroxy-2-(4-methoxyphenyl)ethylphosphonate [(R)-2d]. $[\alpha]_D^{20} = -20.5$ (C=1, $CHCl_3$).

^{1}H NMR: δ 1.31, 1.33, dd (6H, J = 6.4, CH_3), 2.73 br (1H, OH), 2.96 m (2H, $PhCH_2$), 3.79 s (3H, OMe), 3.96 m (1H, CHP), 4.77 m (2H, OCH), 7.02 m (4H, C_6H_4).

^{31}P NMR ($CDCl_3$): 26.8.

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МОДЕЛЬ КОШЛАНДА ЯК МЕТОД АНАЛІЗУ
ФЕРМЕНТАТИВНОЇ РЕАКЦІЇ ДЕРАЦЕМІЗАЦІЇ

Чотириточкова локаційна модель Кошланда застосована для розгляду деяких ферментативних реакцій дерацемізації. Послідовна модель — це теорія, яка описує кооперативність білкових субодиниць. Вона постулює, що конформація білка змінюється з кожним зв'язуванням ліганду, таким чином послідовно змінюючи його спорідненість до ліганду в сусідніх сайтах зв'язування. Коли субстрат зв'язується з активним центром однієї субодиниці ферменту, інші субодиниці активуються. Можливість альтернативного зв'язування як субстрату, так і продукту ферментативної реакції може бути оцінена на основі відомих даних про структуру всіх чотирьох замісників хірального атома та їх відповідності лігандній специфічності відповідних секторів ферменту. Зроблені теоретичні висновки перевірені на прикладі ферментативної дерацемізації деяких гідроксифосфонових кислот. У результаті заміни етоксильних груп в атомі фосфору на ізопропоксильні і збільшення обсягу замісника істотно збільшився енантіомерний надлишок продукту гідролізу гідроксифосфонату. Звідси зроблений висновок, що ключовим критерієм ефективного або неефективного проходження реакції є співвідношення розмірів і ступеня гідрофобності відповідних замісників у асиметричному реакційному центрі.

Ключові слова: *модель Кошланда, ліпази, ферменти, кінетичне розділення, Candida Antarctica lipase.*