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# The influence of cyclomaltooligosaccharides (cyclodextrins) on the enzymatic decomposition of L-phenylalanine catalyzed by phenylalanine ammonia-lyase

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ABSTRACT

Cyclomaltohexaose (α-cyclodextrin) and cyclomaltoheptaose (β-cyclodextrin) as well as their four methyl ether derivatives, that is, hexakis(2,3-di-*O*-methyl)cyclomaltohexaose, hexakis(2,3,6-tri-*O*-methyl)cyclomaltohexaose, heptakis(2,3-di-*O*-methyl)cyclomaltoheptaose, and heptakis(2,3,6-tri-*O*-methyl)cyclomaltoheptaose were investigated as the additives in the course of enzymatic decomposition of L-phenylalanine catalyzed by phenylalanine ammonia-lyase. Only a few of those additives behaved like classical inhibitors of the enzymatic reaction under investigation because the values of the Michaelis constants that were obtained, as well as the maximum velocity values depended mostly atypically on the concentrations of those additives. In most cases cyclodextrins caused mixed inhibition, both competitive and noncompetitive, but they also acted as activators for selected concentrations. This atypical behaviour of cyclodextrins is caused by three different and independent effects. The inhibitory effect of cyclodextrins is connected with the decrease of substrate concentration and unfavourable influence on the flexibility of the enzyme molecules. On the other hand, the activating effect is connected with the decrease of product concentration (the product is an inhibitor of the enzymatic reaction under investigation). All these effects are caused by the ability of the cyclodextrins to form inclusion complexes.


## 1. Introduction

Cyclomaltooligosaccharides (cyclodextrins, CDs) have found crucial applications in the pharmaceutical, cosmetic and food industries due to their ability to form inclusion complexes with a wide range of compounds.[1–6] Before applying CDs in a specific industrial trial, they should first be investigated in basic studies. The very promising basic studies focused on CDs are their applications in enzymology. CDs have served in enzymology as additives added in the course of enzymatic reactions,[7–10] and the substances used for co-lyophilization of the enzymes,[11,12] as well as the covalent modifiers of enzyme molecules.[13] For example, CDs have been successfully used in microencapsulation of the phenylalanine ammonia-lyase enzyme (PAL).[14] The oral administration of the microencapsulated PAL is being considered as an efficient and comfortable treatment in phenylketonuria.[14–16] CDs have been found as the most potent substances of all compounds under investigation in the cited paper for preventing PAL degradation in the stomach.[14] Such an observation prompted us to investigate the influence of native and methyl ether derivatives of CDs as additives in the decomposition of L-phenylalanine (Phe) catalyzed by PAL. This investigation is also the continuation of our previous studies focused on the role of native and different ether derivatives of CDs on the biotransformations catalyzed by tyrosine phenol-lyase[7] and tryptophan indole-lyase.[8,9]

## 2. Results and discussion

In the course of the enzymatic decomposition of Phe catalyzed by PAL, native and selectively O-methylated cyclodextrin derivatives (Scheme 1) were added to evaluate their influence on this biotransformation. PAL catalyzes the decomposition of Phe to the corresponding cinnamic acid and ammonia[17,18] (Scheme 2). Although PAL can catalyze the reverse reaction to the above-described decomposition, that is, the synthesis of Phe from cinnamic acid, this reverse reaction could not be performed in this experiment. In order to obtain Phe from cinnamic acid and ammonia, different experimental conditions are needed, in contrast to the decomposition of Phe.[19,20] Therefore, the studied reaction can be classified as an irreversible one.

The CD molecules can include either the substrate (Phe)[21–28] or the product (cinnamic acid)[29–31] of the reaction under investigation. If the CD molecules form the inclusion complexes with the

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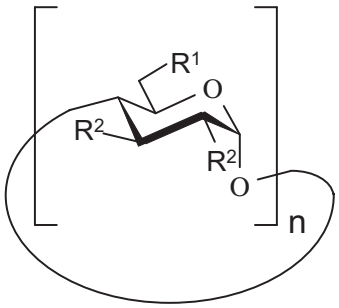


| Compound | n | $R^1$ | $R^2$ |
|---|---|---|---|
| α-CD | 6 | OH | OH |
| β-CD | 7 | OH | OH |
| DM-α-CD | 6 | OH | $OCH_3$ |
| DM-β-CD | 7 | OH | $OCH_3$ |
| TM-α-CD | 6 | $OCH_3$ | $OCH_3$ |
| TM-β-CD | 7 | $OCH_3$ | $OCH_3$ |

**Scheme 1.** Structural formulas of native and methyl ether derivatives of cyclodextrins added in the course of decomposition of L-phenylalanine (Phe) catalyzed by phenylalanine ammonia-lyase (PAL).


**Scheme 2.** Decomposition of L-phenylalanine (Phe) catalyzed by phenylalanine ammonia-lyase (PAL).

substrate, the inhibition should be observed because of decreasing concentration of the substrate. On the other hand, the formation of inclusion complexes between CDs and the product of the enzymatic reaction should activate this process. Since cinnamic acid is an inhibitor of this enzymatic process,[31,32] the decrease in its concentration by inclusion complex formation should cause the activating effect. Apart from the formation of inclusion complexes between the substrate and the product of this biotransformation, the influence of CDs on PAL molecules should be expected, also. According to the studies performed by Shah and D'mello,[14] CD derivatives fully protected PAL against any loss in activity during emulsification. This phenomenon can be explained following the observation that CDs physically interact with hydrophobic amino acids on the surface of the proteins.[33] Such interaction should maintain the activity of PAL during the unfavourable influence of emulsification.

A possible effect of the introduction of buffer solution on the experimental results should also be mentioned. According to the paper by Song and Bai,[34] inorganic ions help CDs with stronger binding of amino acids as well as they increase the molecular recognition of CDs towards enantiomers of amino acids. Therefore, the formation of stronger host–guest complexes between CDs and Phe can be expected in buffer solution.

The kinetic parameters that were obtained in this study are collected in Table 1, whereas the dependences of these parameters on CD concentration are shown in Figures 1 and 2. Table 1 contains the values of the Michaelis constants ($K'_m$) and the inverse maximum velocities ($1/V'_{max}$) in the presence and absence of CDs. Figures 1 and 2 depict the $K'_m$ and $1/V'_{max}$ values versus the concentrations of a particular CD, respectively.

In general, the Michaelis constant ($K_m$) equals the substrate concentration ([S]) at which the rate of enzymatic reaction ($v_0$) reaches its half-maximum value ($\frac{1}{2}V_{max}$). The correlation of those kinetic parameters is depicted in the Michaelis–Menten equation:[35]

$$v_0 = \frac{V_{max} \cdot [S]}{K_m + [S]} \left(\text{after transforming } \frac{1}{v_0} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}\right).$$

From this equation one can calculate kinetic parameters ($K_m$ and $1/V_{max}$) from experimental data which result in a Lineweaver–Burk plot.

According to the results obtained (Table 1 and Figs. 1 and 2), only a few of the CDs under investigation behaved like typical inhibitors of the enzymatic decomposition of Phe. For typical inhibitors, the correlations of $K'_m$ and $1/V'_{max}$ against their concentration should be linear, and the values of the slopes of these dependences should be positive. In this study, only for α-CD, DM-α-CD and DM-β-CD the dependences of $K'_m$ on concentration fulfil this requirement. Therefore, only in this case it is possible to calculate the inhibition constants. According to the following equation:[35]

$$K_i^1 = [CD]/(K'_m/K_m - 1),$$

where $K_i^1$ denotes the competitive inhibition constant, [CD] CD concentration, $K'_m$ and $K_m$ the Michaelis constant with the presence and absence of the inhibitor, respectively, the $K_i^1$ values for α-CD, DM-α-CD and DM-β-CD equal 11 ± 1, 0.57 ± 0.08, and 0.39 ± 0.08 mM, respectively. These values indicate that DM-α-CD and DM-β-CD are strong competitive inhibitors, whereas α-CD is a weak competitive inhibitor of the enzymatic decomposition of Phe. Although the dependences of $K'_m$ versus concentration of TM-α-CD and TM-β-CD are also linear, this time the values of the slopes of these lines are negative. However, these CD derivatives are competitive inhibitors of the reaction under investigation, because the $K'_m$ values are higher in comparison with the $K_m$ value. The higher is the concentration of TM-α-CD and TM-β-CD, the lower is $K'_m$ value; therefore, the inhibitory effect of these CD derivatives decreases with the increase in their concentration. The most divergent behaviour was exhibited by β-CD because at its concentrations of 1 and 3 mM, it is a competitive inhibitor, whereas at a concentration of 2 mM it acts as an activator.

In the case of dependences of $1/V'_{max}$ versus concentration of CDs (Fig. 2), no linear correlations were observed for all CDs. Therefore, the calculations of the noncompetitive inhibition constants could not be performed in this case. All CDs, except DM-α-CD for selected concentrations, acted as noncompetitive inhibitors of the decomposition of Phe because the $1/V'_{max}$ values were higher than the $1/V_{max}$ value ($1/V_{max}$ stands for inverse maximum velocity in the absence of CDs). For DM-α-CD concentrations equal to 2 and 3 mM, this CD derivative is the activator of the process under investigation because of lower $1/V'_{max}$ values than the $1/V_{max}$ value. On the other hand, DM-α-CD, for its concentration equal to 1 mM, behaves as a noncompetitive inhibitor. Interestingly, the highest $1/V'_{max}$ values were obtained for β-CD at its concentration equal to 1 and 2 mM. The higher the $1/V'_{max}$ value is, the stronger the inhibition that is observed; therefore, β-CD is the strongest noncompetitive inhibitor in this study.

The above-reported effects of CDs are very atypical, but it should be kept in mind that CDs played a triple role in this

**Table 1**
The values of Michaelis constants and inverse maximum velocities for native and methyl ether derivatives of cyclodextrins studied in PAL-catalyzed reaction in the presence ($K'_m$ and $1/V'_{max}$, respectively) and absence ($K_m$ and $1/V_{max}$, respectively) of particular CD

| Kinetic parameters | $K'_m$ (mM) | $1/V'_{max}$ (min × μM$^{-1}$) | $K'_m$ (mM) | $1/V'_{max}$ (min × μM$^{-1}$) | $K'_m$ (mM) | $1/V'_{max}$ (min × μM$^{-1}$) |
|---|---|---|---|---|---|---|
| CD concentration | 1 mM | | 2 mM | | 3 mM | |
| α-CD | 219 ± 22 | 971 ± 93 | 241 ± 101 | 909 ± 31 | 270 ± 80 | 1070 ± 102 |
| β-CD | 243 ± 11 | 1733 ± 216 | 101 ± 14 | 1916 ± 180 | 262 ± 62 | 885 ± 103 |
| DM-α-CD | 482 ± 31 | 794 ± 102 | 1010 ± 233 | 543 ± 23 | 1781 ± 292 | 546 ± 24 |
| DM-β-CD | 553 ± 32 | 952 ± 54 | 1595 ± 581 | 741 ± 60 | 2565 ± 244 | 735 ± 23 |
| TM-α-CD | 395 ± 33 | 735 ± 48 | 312 ± 41 | 813 ± 21 | 256 ± 28 | 1081 ± 44 |
| TM-β-CD | 309 ± 47 | 1080 ± 35 | 260 ± 15 | 1335 ± 55 | 249 ± 26 | 1453 ± 129 |
| Kinetic parameters | | | $K_m$ (mM) | | | $1/V_{max}$ (min × μM$^{-1}$) |
| Phe | | | 212 ± 81 | | | 662 ± 32 |

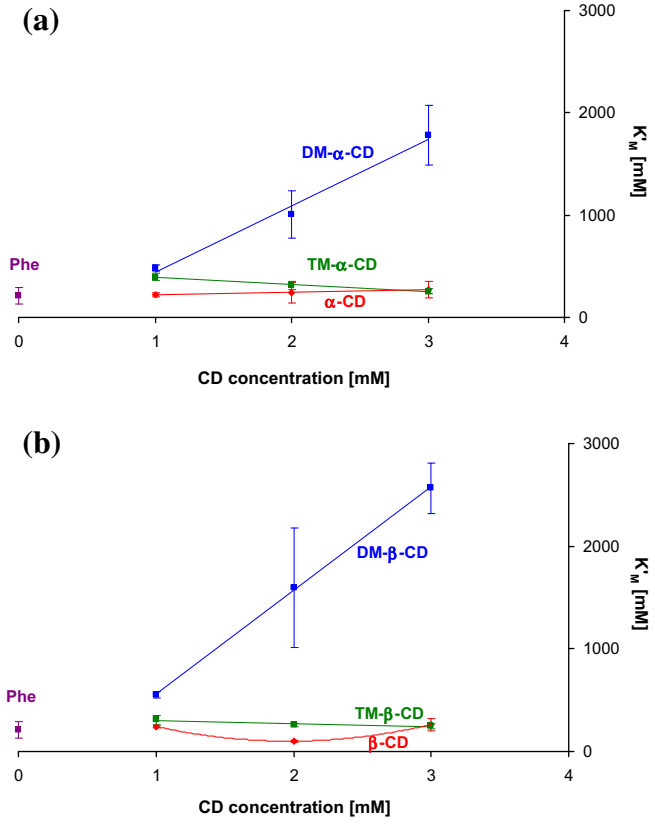


**Figure 1.** The dependence of the Michaelis constants ($K'_m$) on the concentrations of particular (a) α-CD and (b) β-CD derivatives.

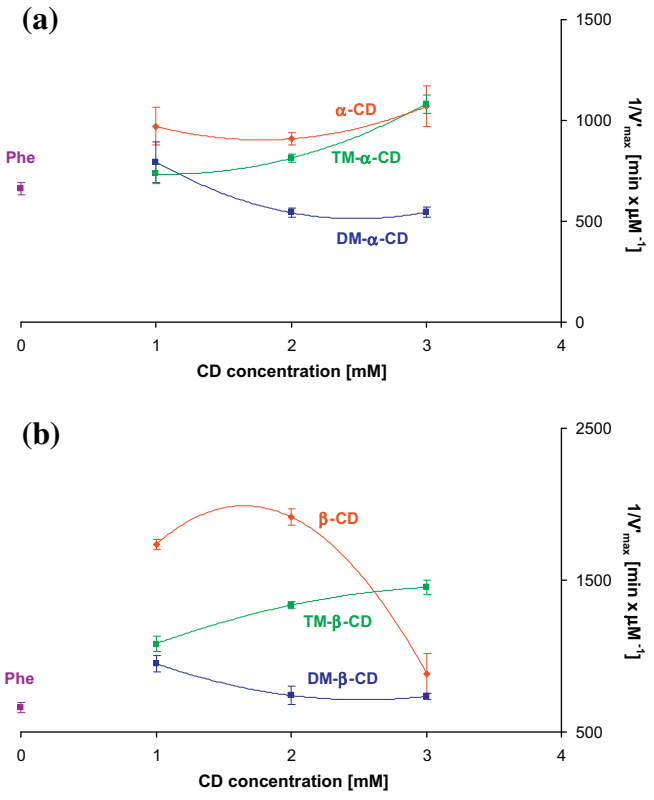


**Figure 2.** The dependence of the inverse maximum velocities ($1/V'_{max}$) on the concentrations of particular (a) α-CD and (b) β-CD derivatives.

experiment. CDs affected the concentrations of both substrate and the product of biotransformation under investigation, and also they interacted with PAL molecules. The higher the CD concentration is, the more CD molecules remained uncomplexed, because there was the priority of formation of inclusion complexes with Phe first of all due to previous incubation with the substrate. Therefore, with the increase in CD concentrations, more and more CD molecules were available for the formation of inclusion complexes also with cinnamic acid, as well as for interactions with PAL protein. Then, at higher CD concentration, CDs should exhibit a more potent activating effect because of entrapping more disturbing cinnamic acid molecules. In the case of CD interactions with PAL protein, the more available free CD molecules should show their real influence on PAL.

The strongest competitive inhibition was observed for DM-α-CD and DM-β-CD; therefore, in this case the strongest effect of all effects considered in this study is the formation of inclusion complexes with Phe. Moreover, we can assume that DM-α-CD and DM-β-CD form the strongest inclusion complexes with Phe, whereas the rest of CDs form weak host–guest complexes with the substrate. Supporting literature[28] indicates that α-CD and β-CD form rather weak complexes with Phe because the association constants, $K_a$, for their complexes are equal to 7.9 ± 1.7 and 3.0 ± 3.3 dm$^3$ mol$^{-1}$ ($\log K_a = 0.90 \pm 0.24$ and $0.48 \pm 0.52$), respectively.

According to the results obtained, we did not observe any spectacular activation of the PAL-catalyzed decomposition of Phe; therefore, we can assume that CDs form weak complexes with cinnamic acid. Despite the fact that CDs form weak complexes with cinnamic acid, β-CD at a concentration of 2 mM acted as an activator. A similar influence of β-CD on the PAL-catalyzed reaction was observed earlier;[31] however, in the cited paper the reverse reaction was studied.

Noncompetitive inhibitors exert an unfavourable effect on the enzyme protein, and therefore the enzyme has a problem in

recognizing the substrate. Almost in all cases, CDs were noncompetitive inhibitors of PAL. With increasing CD concentration, only TM-α-CD and TM-β-CD increased their inhibitory properties. Therefore, we can expect that TM-α-CD and TM-β-CD exhibited definitely unfavourable interactions with PAL protein. On the other hand, the specific dependences of $1/V'_{max}$ values versus DM-α-CD and DM-β-CD concentration can be caused by two different effects of these CDs on PAL. As mentioned above, DM-α-CD and DM-β-CD form the strongest inclusion complexes with Phe of all CDs examined; therefore, we can assume that in this case there is the highest concentration of complexed Phe. With an increasing concentration of DM-α-CD and DM-β-CD, the ratio of complexed to free CD molecules decreases. Probably host–guest complexes of Phe with DM-α-CD and DM-β-CD unfavourably interact with PAL protein, whereas free DM-α-CD and DM-β-CD help PAL in molecular recognition of the substrate. Therefore, probably for DM-α-CD concentrations of 2 and 3 mM, the activation of PAL was observed.

## 3. Experimental

### 3.1. Materials

Cyclomaltohexaose (α-CD) and cyclomaltoheptaose (β-CD), PAL (phenylalanine ammonia-lyase from *Rhodotorula glutinis*, solution in 60% glycerol, 10 U/mL, EC 4.3.1.5), L-phenylalanine (Phe) were purchased from Sigma–Aldrich Chemie GmbH.

### 3.2. CD derivatives

#### 3.2.1. Hexakis(2,3-di-*O*-methyl)cyclomaltohexaose (DM-α-CD)

The synthesis and full analytical data for DM-α-CD have been reported earlier.[9]

#### 3.2.2. Heptakis(2,3-di-*O*-methyl)cyclomaltoheptaose (DM-β-CD)

The synthesis and full analytical data for DM-β-CD have been reported earlier.[9]

#### 3.2.3. Hexakis(2,3,6-tri-*O*-methyl)cyclomaltohexaose (TM-α-CD)

The synthesis of TM-α-CD has been reported earlier.[8] White amorphous powder; yield 62%; $[\alpha]_D^{20}$ +163.0° (*c* 1.5; $CHCl_3$), lit.[36] $[\alpha]_D^{22}$ +164° (*c* 1.1; $CHCl_3$); $R_f$ 0.50 (2:1 chloroform–acetone). $^1H$ NMR (300 MHz, $CDCl_3$): $\delta$ 5.05 (d, 6H, $J_{H\text{-}1,H\text{-}2}$ 3.3 Hz, 6H-1); 3.86–3.74 (m, 12H, 6H-6a,6H-5); 3.71–3.68 (m, 6H, 6H-6b); 3.64 (s, 18H, 6 × C-3–OC$H_3$); 3.61–3.50 (m, 12H, 6H-4,6H-3); 3.49 (s, 18H, 6 × C-2–OC$H_3$); 3.40 (s, 18H, 6 × C-6–OC$H_3$); 3.17 (dd, 6H, $J_{H\text{-}2,H\text{-}3}$ 9.3 Hz, 6H-2); $^{13}C$ NMR (75 MHz, $CDCl_3$): $\delta$ 100.34 (C-1); 82.67 (C-4); 82.40 (C-2); 81.42 (C-3); 71.66 (C-6); 71.42 (C-5); 62.02 (C-3–O$CH_3$); 59.20 (C-6–O$CH_3$); 58.04 (C-2–O$CH_3$). ESIMS: calcd for $C_{54}H_{96}O_{30}$, *m/z* 1225.3; found, *m/z* 1248.9 $[M+Na]^+$.

#### 3.2.4. Heptakis(2,3,6-tri-*O*-methyl)cyclomaltoheptaose (TM-β-CD)

The synthesis of TM-β-CD has been reported earlier.[8] Froth; yield 79%; $[\alpha]_D^{20}$ +144.6° (*c* 1.0; $CHCl_3$), lit.[37] $[\alpha]_D$ +158° (*c* 1.4; $CHCl_3$); $R_f$ 0.62 (10:1 chloroform–MeOH). $^1H$ NMR (300 MHz, $CDCl_3$): $\delta$ 5.13 (d, 7H, $J_{H\text{-}1,H\text{-}2}$ 3.3 Hz, 7H-1); 3.88–3.80 (m, 14H, 7H-6a,7H-5); 3.65 (s, 21H, 7 × C-3–OC$H_3$); 3.62–3.48 (m, 21H, 7H-4,7H-6b,7H-3); 3.51 (s, 21H, 7 × C-2–OC$H_3$); 3.39 (s, 21H, 7 × C-6–OC$H_3$); 3.19 (dd, 7H, $J_{H\text{-}2,H\text{-}3}$ 9.6 Hz, 7H-2); $^{13}C$ NMR (75 MHz, $CDCl_3$): $\delta$ 99.16 (C-1); 82.25 (C-2); 81.96 (C-3); 80.49 (C-4); 71.61 (C-6); 71.12 (C-5); 61.65 (C-3–O$CH_3$); 59.16 (C-6–O$CH_3$); 58.71 (C-2–O$CH_3$). ESIMS: calcd for $C_{63}H_{112}O_{35}$, *m/z* 1429.6; found, *m/z* 1452.0 $[M+Na]^+$.

### 3.3. Analyses

$^1H$ and $^{13}C$ NMR spectra for $CDCl_3$ solutions were recorded at 300 and 75 MHz, respectively, on a Varian VNMRS-300 spectrometer. The 2D experiments (COSY, HSQC, and HMBC) were run using standard Varian software. Optical rotation values were measured on a Perkin–Elmer 241 polarimeter at a wavelength of 589 nm. Molecular peaks in mass spectrometry were collected on an AMD 604 Intectra spectrometer.

### 3.4. Methods of kinetic data determination

Phe for measurements was incubated with a proper excess of each CD derivative. Incubation was carried out overnight at room temperature. A typical assay contained borate buffer at pH 8.8, 2.5 μL of 10 U/mL PAL, various amounts of the particular CD derivative (1, 2 or 3 mM), and various amounts of Phe (0.08, 0.12, 0.2, 0.32, 0.4 or 0.8 mM). Six assays of different concentrations of Phe were applied per one experiment. The substrate concentration varied between the experiments of one series, whereas the CD total concentration remained unchanged. The CDs concentrations varied between different experimental series. The concentration of CDs was always in proper excess in comparison with substrate. Each experiment was repeated at least three times. The increase of absorbance was measured at a wavelength of 290.0 nm on a 1202 Shimadzu UV–vis spectrophotometer during the time of reaction. The experiments were performed at room temperature (20 °C). The Lineweaver–Burk plots were used to determine the intercepts (inverse maximum velocity), and slopes (ratio of the Michaelis constant per maximum velocity) using the least-squares method.

## 4. Conclusions

The aim of this paper was to evaluate the influence of native and some methyl ether derivatives of CDs on the enzymatic decomposition of Phe catalyzed by PAL. The oral administration of microencapsulated PAL was considered as a very convenient treatment in phenylketonuria, and CD derivatives were found as the substances which preserved PAL activity during the process of preparation of such PAL form.[14] In the cited paper, hydroxypropylcyclomaltooctaose and hydroxypropylcyclomaltoheptaose served as efficient protectors of PAL. However, in our studies the CDs behaved mostly as both competitive and noncompetitive inhibitors. Only for selected concentrations of β-CD and DM-α-CD, were they PAL activators; therefore, only these compounds of all CD derivatives considered in this study can serve as protectors of PAL. Nevertheless, the rest of the CDs under investigation, that is, α-CD, TM-α-CD, DM-β-CD and TM-β-CD cannot be considered as the additives to PAL-based drugs towards phenylketonuria. Although the results obtained are mostly atypical for classical inhibitors or activators, it should be stressed that CDs played a triple role in these cases. CDs can affect the concentrations of both substrate and the product of biotransformation under investigation, and also they can interact with the enzyme protein.

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