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Interactions between *p*-hydroxybenzoic acid esters and hydroxypropyl-β-cyclodextrin and their antimicrobial effect against *Candida albicans*

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Summary

The interactions between hydroxypropyl-β-cyclodextrin (HP-β-CD) and a series of PHB esters were studied in aqueous solution by solubility isotherms and in the solid state by thermal analysis (DSC) and X-ray diffractometry. Calculated complex constants show different stabilities of the inclusion complexes formed. The antimicrobial effect against *Candida albicans* was tested and MIC values were ascertained. It was found that the antimicrobial activity of the preservatives against the test organism was reduced in the presence of HP-β-CD. QSAR analyses were made to describe this effect by physicochemical parameters such as lipophilicity or complex stability constants. The results suggest that the degree of inactivation is completely dependent on the complex bonded fraction of the PHB ester.

Introduction

Hydroxypropyl-β-cyclodextrin is a cyclic oligosaccharide consisting of seven glucose units which are modified by treatment of β-cyclodextrin with propylene oxide. The ability to form inclusion complexes with many lipophilic drug molecules effects the physicochemical or biopharmaceutical properties of the included substances. This results in increased solubility (Müller and Brauns, 1983), increased stability (Backensfeld et al., 1991) or enhancement of bioavailability (Uekama and Otagiri, 1987) of the included drug molecule.

Aqueous solutions containing cyclodextrins may show the risk of microbial contamination. Therefore, the addition of preservatives can be considered. Today PHB esters are frequently used for preserving injection formulations and liquids for oral application (Wallhäußer, 1985). The PHB esters are highly lipophilic substances; interactions with cyclodextrins seem very probable. Interactions between a series of PHB esters with α-CD and β-CD have been investigated (Uekama et al., 1980). Decreased antimicrobial activity was found against *Candida albicans* and other mi-

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croorganisms in the presence of both types of cyclodextrin. Loftsson and co-workers (1992) determined an increase in solubility of the HP-β-CD complexes of methyl and propyl parabens.

In this study, the interactions between PHB esters and HP-β-CD in aqueous solutions were investigated. In particular, the determination of the antimicrobial activity against *C. albicans* was performed.

Materials and Methods

Hydroxypropyl-β-cyclodextrin (average degree of substitution: 0.49) was obtained from Janssen (Beerse, Belgium), while *p*-hydroxybenzoic acid esters were a gift from Nipa Laboratorien (Norderstedt, Germany) except the amyl ester which was obtained from Sigma Chemie GmbH (Deisenhofen, Germany). All substances were used without further purification.

Sabouraud 2% dextrose-agar, Sabouraud 2% dextrose-broth and casein-peptone soymeal-peptone broth were purchased from Merck (Darmstadt, Germany).

The solvents were of HPLC grade, all other chemicals were commercially available products of reagent grade.

HPLC analyses

Quantitative determinations of *p*-hydroxybenzoic acid esters were carried out by a high-performance liquid chromatographic system consisting of a Gynkotek High Precision Pump Model 300 (Gynkotek, München, Germany), an Automatic Sampler 360 (Kontron Instruments, Zürich, Switzerland), a Shandon Hypersil ODS 5 μm column (4.6 × 250 mm) (Runcorn, U.K.) and a UV spectrophotometric detector SPD-6A Module (Shimadzu, Kyoto, Japan). All determinations were performed with acetonitrile-water mixtures as mobile phase; for all detections the wavelength of 256 nm was adjusted.

Solubility studies

Solubility measurements were carried out according to the method of Higuchi and Connors (1965). An excess of the antimicrobial substance was mixed with 10 ml of phosphate buffer pH 7.4 containing various amounts of HP-β-CD (2–12% w/v). The suspensions were shaken at 25°C for at least 7 days. After filtration through a 0.2 μm cellulose acetate membrane filter (Sartorius, Göttingen, Germany) the saturated concentration of PHB ester was analyzed by HPLC.

X-ray diffractometry

Powder X-ray diffractometry was carried out using a Stoe Powder Diffractometer System (Stoe & Cie, Darmstadt, Germany).

Differential scanning calorimetry (DSC)

Samples, approx. 6 mg, were examined using a 7 Series Thermal Analysis System (Perkin-Elmer, Norwalk, U.S.A.) at a scanning speed of 10 K min^{-1} in the range 323–473 K.

Testing of antimicrobiological efficiency

A series of PHB ester solutions was prepared, with and without addition of HP-β-CD in equimolar proportion to the preserving agent (medium: Sabouraud broth/phosphate buffer pH 7.4 1:10). After sterile filtration through 0.2 μm cellulose acetate membrane the content of antimicrobial agent in the stock solution was analyzed by HPLC. The stocks were diluted with broth in steps of 10% to prepare the test solutions.

C. albicans (ATCC 10231) was used as test organism. After cultivation in casein-peptone soymeal-peptone broth (CASO broth) at 37°C for 72 h the inoculum was adjusted by dilution with CASO broth to about 10^5 organisms per ml. 30 μl of inoculum was added to each 3.0 ml of test solutions. Each test was carried out with duplicate samples.

After incubation at 37°C for 72 h the test solutions were diluted with inactivation liquid (1% Polysorbate 80 in CASO broth) and aliquots were transferred on Sabouraud agar plates containing 1% Polysorbate 80. The growth of *C. albicans* expressed in numbers of colony forming units (CFU) was determined by the plate count method.

Results and Discussion

Solubility curves

Fig. 1 shows the enhanced solubility of some PHB esters in phosphate buffer pH 7.4 after addition of various amounts of HP-β-CD. For each substance the isotherm type A_L is found which corresponds to a linear correlation between increasing solubility of PHB esters and the HP-β-CD concentration ($r^2 > 0.999$).

The solubility of the tested substances in buffer solution decreased with increasing chain length from methyl ester (2.24 mg/ml) to benzyl ester (28.2 μg/ml) whereas the extent of solubilization is inverted. The solutions containing 12% HP-β-CD show an increase in solubility of 6-fold (12.7 mg/ml) for the methyl derivative but 308-fold (8.7 mg/ml) for the benzyl ester. The linear isotherm is generally ascribed to the formation of a 1:1 complex when the slope is less than unity. The apparent complex stability constant ($K_{1:1}$) can be calculated according to the formula

$$K_{1:1} = \frac{\text{slope}}{(1 - \text{slope}) \times S_0}$$

where S_0 denotes the intrinsic solubility of the substance in buffer solution. In Table 1 the calculated complex constants ($K_{1:1}$) are listed. The complex stability rises with increasing chain length. This is in agreement with observations that much more lipophilic substances are preferred for inclusion in the lipophilic cavity of the cyclodextrin ring.

TABLE 1

Investigation of PHB esters and their HP-β-CD complexes: Some physicochemical data and minimal inhibition concentrations against Candida albicans

PHB ester	$K_{(1:1)}$ ($l\ mol^{-1}$)	log *P*	MIC (μg/ml)	$MIC_{1:1\ complex}$ (μg/ml)
Methyl	969	1.85	750.2	3300.7
Ethyl	948	2.33	519.7	1900
Propyl	1548	2.87	200.0	579.9
Butyl	3352	3.44	70.0	139.9
Amyl	4760	4.04 (calc.)	55.0	130.0
Benzyl	6039	3.56	65.0	120.1

DSC and X-ray diffractometry

Further evidence of complex formation was obtained from the DSC curves (Fig. 2). The endothermic peak at 95°C equivalent to the propyl ester disappeared in the inclusion complex systems prepared by spray-drying and freeze-drying of the propyl paraben-HP-β-CD containing solutions (1:2 mol). In contrast to these results the peak of propyl paraben is present in the physical mixture. Complex formation has not completely taken place in this formulation.

Similar results are obtained by X-ray diffraction analysis of powder samples (Fig. 3). The

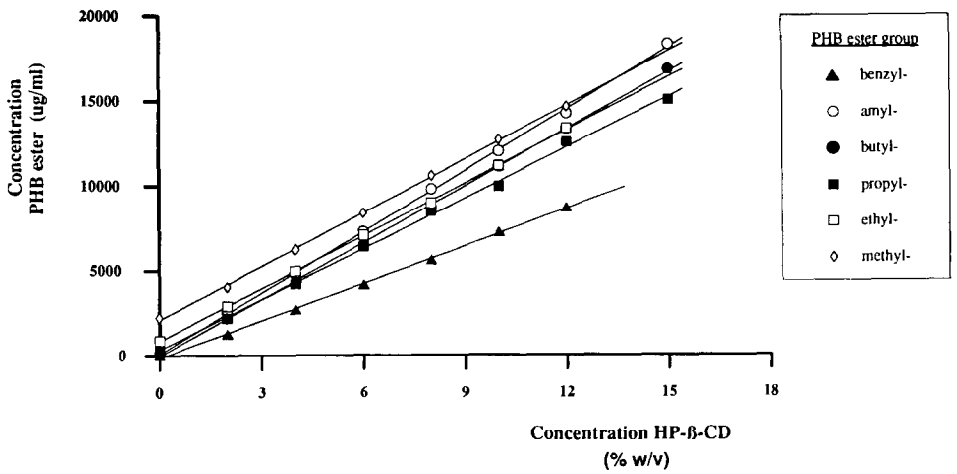

Fig. 1. Solubility isotherms of PHB esters with HP-β-CD.

complex systems revealed no crystalline parts in the diffraction patterns which indicates that the substance is completely bonded to the cyclodextrin molecule. On the other hand, peaks are still observed from the physical mixture.

Antimicrobial testing

The effect of HP-β-CD on growth of *C. albicans* was investigated. Only slight changes in the growth behaviour up to a concentration of 40% HP-β-CD in Sabouraud broth-buffer medium (1:10) compared to the control without CD were seen. The observed deviations may be due to biological variations. It is known that *C. albicans* is, like other yeasts, a very carbophilic organism. To determine whether there is any utilization of glucose resulting from enzymatic degradation of the cyclodextrin molecule the experiment was repeated with HP-β-CD in distilled water. No difference in growth between HP-β-CD containing solutions and distilled water alone was found. It can be concluded that *C. albicans* did not use the cyclodextrin molecule for nutrition within 5 days.

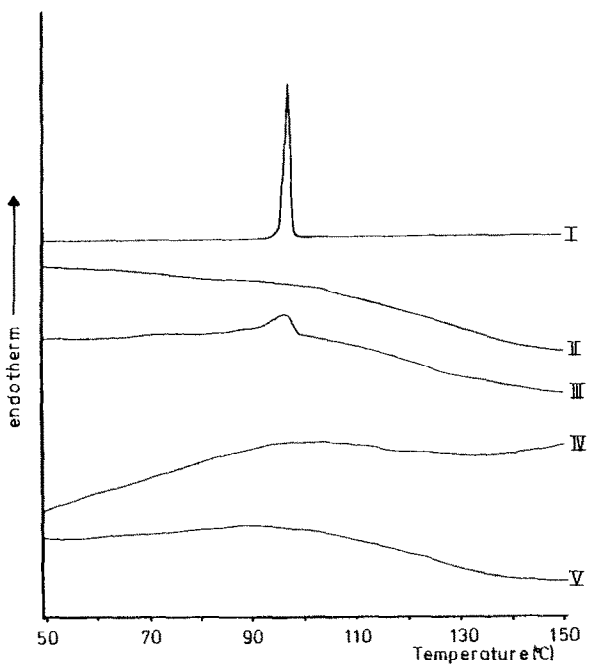

Fig. 2. Differential scanning calorimetry of: propyl paraben (I); HP-β-CD (II); the physical mixture of propyl paraben and HP-β-CD (1:2 mol) (III); the spray-dried mixture of propyl paraben and HP-β-CD (1:2 mol) (IV); the freeze-dried mixture of propyl paraben and HP-β-CD (V).

The MIC data of the homologous series of PHB esters and their interactions with equimolar amounts of HP-β-CD are presented in Table 1. The solutions containing HP-β-CD show a partial inactivation of the preserving agent. Higher concentrations of ester are required for the same antimicrobial effect. This is due to the inclusion of the preservative in the cyclodextrin molecule. Only the free, unbonded part in solution is active against the microorganism.

High concentrations of preservatives may cause some toxic effects to the user. If any pharmaceutical preparation containing HP-β-CD is preserved with PHB ester the total amount of ester must be increased 2–4-fold. The risk of further side effects will be more probable especially as these substances are noted for their high allergic potential (Wallhäußer, 1988).

Quantitative structure activity relationship (QSAR) analysis

As seen in Fig. 4 there is a strong linear relationship between MIC values and the lipophilicity of the PHB esters as well as their complexes with HP-β-CD. Log P values were taken as a measure of the degree of lipophilicity where P denotes the octanol-phosphate buffer pH 7.4 partition coefficient. Log P values of PHB esters have been measured with the HPLC procedure described by Unger et al. (1978). The log P value for the amyl ester was calculated according to additive principles (log $P_{\text{amyl ester}}$ = log $P_{\text{butyl ester}} + \pi_{\text{methyl}} = 3.54 + 0.50 = 4.04$) because the lipophilicity is too high to use the HPLC method (Seydel and Schaper, 1979).

It is evident that in the presence of HP-β-CD higher concentrations of preservative are necessary to achieve the same biological effect. This behaviour can be described by Eqns 1 and 2 in Table 2. The results for substances without addition of HP-β-CD are in good agreement with data reported by Hansch and Dunn (1972) (Eqn 3 in Table 2) and with Eqn 4 calculated by Hansch and Lien (1971) who also measured the antimicrobial effect of PHB esters against *C. albicans*. The intercepts which are determined by the sen-

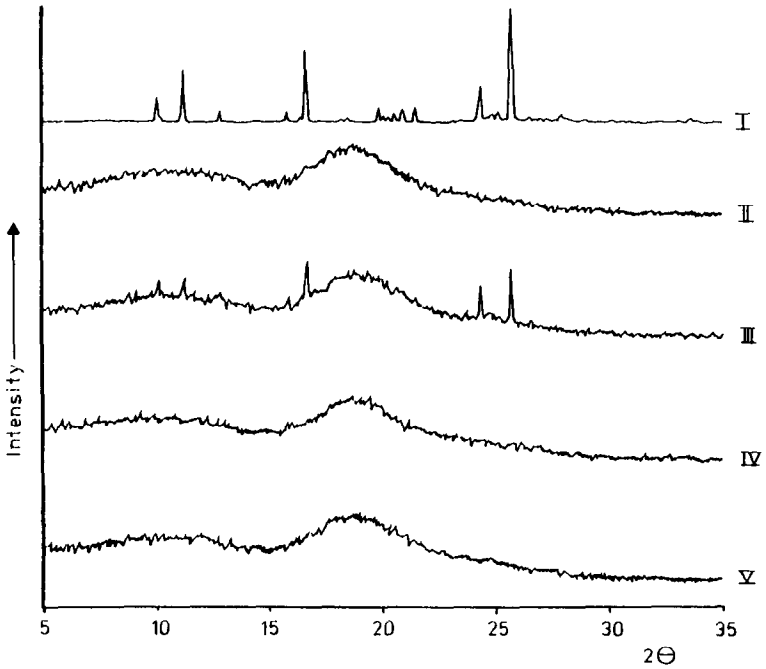

Fig. 3. Powder X-ray diffraction patterns of: propyl paraben (I); HP-β-CD (II); the physical mixture of propyl paraben and HP-β-CD (1:2 mol) (III); the spray-dried mixture of propyl paraben and HP-β-CD (1:2 mol) (IV); the freeze-dried mixture of propyl paraben and HP-β-CD (V).

sitivity of the biological system show great differences (but there is no significant difference at the 95% level). The experimental conditions in measuring MIC values are very different especially concerning the starting number of CFU, the kind of medium and the test period. It will be difficult to compare absolute MIC values from different studies.

A linear relationship was also found by Shibasaki (1969) up to a chain length of 7–8

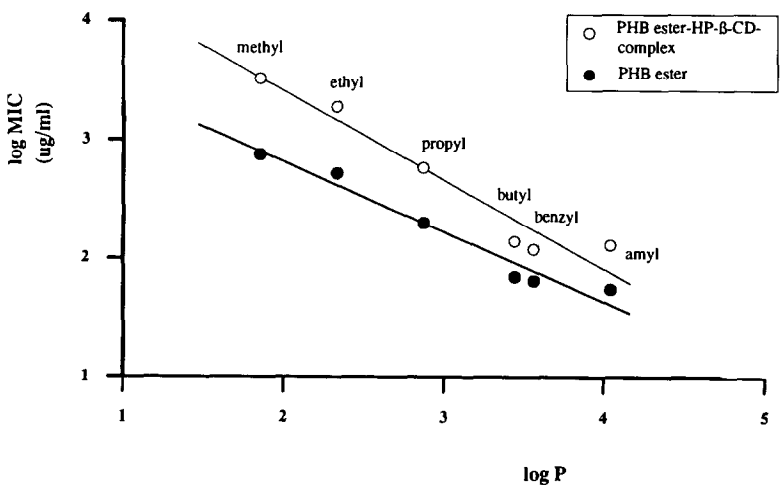

Fig. 4. Effect of partition coefficient of PHB esters and their HP-β-CD complexes on MIC against *Candida albicans*.

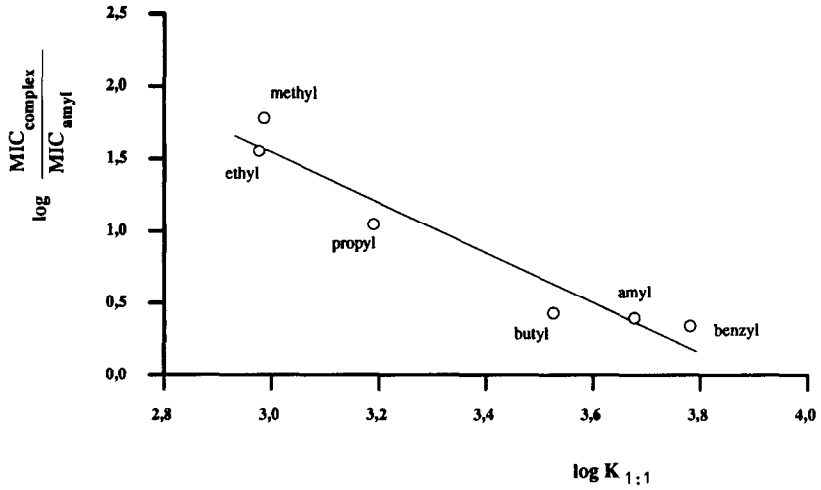

Fig. 5. Correlation between complex constants and loss of antimicrobial activity of PHB ester-HP-β-CD-complexes against *Candida albicans*.

carbon atoms for bacteria and yeasts: the point of highest activity will be reached at this chain length. This is the reason why others suggest a parabolic relationship between log 1/MIC and log *P* (Hansch et al., 1972).

Following the QSAR methods the effect of inactivation of parabens by complex formation with HP-β-CD can be expressed as shown in Figs 5 and 6. In these plots the MIC values of the complexes are standardized because of the very different activities of the PHB esters. The amyl ester with the highest antimicrobial activity is selected as reference. As well as lipophilicity the complex constants can describe the extent of inactivation in a linear relationship (Eqns 5 and 6 in Table 2). The degree of inactivation rises with decreasing activity of the preserving agent if equimolar concentrations of HP-β-CD are present. Under these conditions, i.e., equimolar ratio of substances, it will be advantageous to select substances with higher activities for preservation of the cyclodextrin containing systems. However,

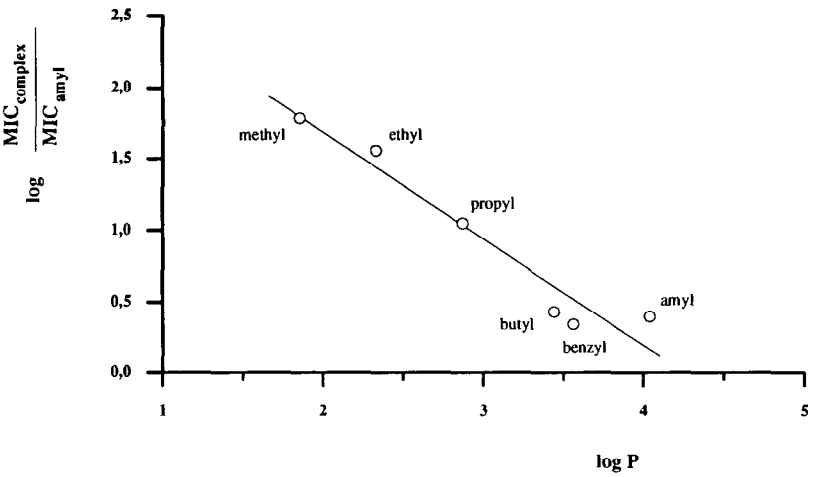

Fig. 6. Correlation between lipophilicity and loss of antimicrobial activity of PHB ester-HP-β-CD-complexes against *Candida albicans*.

TABLE 2

QSAR equations of the antifungal activity of some PHB esters and their HP-β-CD complexes against Candida albicans

No.	Linear equation	n	r	s	Fig. no.	Reference
I	$\log(1/\mathrm{MIC}) = 0.663 \log P\ (\pm 0.186) - 1.943\ (\pm 0.576)$	6	0.980	0.124	4	
II	$\log(1/\mathrm{MIC}_{1:1\ \mathrm{complex}}) = 0.833 \log P\ (\pm 0.294) - 2.891\ (\pm 0.915)$	6	0.980	0.115	4	
III	$\log(1/\mathrm{MIC}) = 0.690 \log P\ (\pm 0.240) + 0.616\ (\pm 0.880)$	7	0.956	0.251	–	Hansch/Dunn (1972)
IV	$\log(1/\mathrm{MIC}) = 0.704 \log P\ (\pm 0.20) + 0.954\ (\pm 0.62)$	7	0.971	0.205	–	Hansch/Lien (1971)
V	$\log(\mathrm{MIC}_{1:1\ \mathrm{complex}}/\mathrm{MIC}_{\mathrm{amyl}}) = -1.913 \log K_{(1:1)}\ (\pm 0.716) + 7.386\ (\pm 2.413)$	6	0.966	0.202	5	
VI	$\log(\mathrm{MIC}_{1:1\ \mathrm{complex}}/\mathrm{MIC}_{\mathrm{amyl}}) = -0.833 \log P(\pm 0.295) + 3.475\ (\pm 0.916)$	6	0.980	0.156	6	
VII	$\mathrm{MIC}_{1:1\ \mathrm{complex}}/\mathrm{MIC} = -9.014 \times 10^{-2}\ \alpha\ (\pm 0.029) + 6.084\ (\pm 1.066)$	6	0.975	0.248	7	

95% confidence intervals are given in parentheses; n, number of tested substances; r, coefficient of correlation; s, standard deviation).

if the experiments are performed with a large molar excess of cyclodextrin the results will be the reverse of this situation. In this case a preservative with low activity must be selected in order to minimize the required excess of ester.

This has also been shown by Uekama et al. (1980) who ascertained an increase of inactivation with increasing $K_{(1:1)}$ values for the paraben-α-cyclodextrin complexes. This was deduced from their fixed α-cyclodextrin concentration (10^{-2} mol/l) in all test solutions which led to a great excess of complexing agent. Highly active substances showing low MIC values are more inactivated than poorly active substances. The concentration of cyclodextrin derivative in our test solutions is adjusted to be in equimolar proportion to the preservative, therefore the results are independent of the great differences in the activities of the PHB esters.

Further insight into complex formation of PHB esters can be obtained by calculation of the degree of dissociation α of their HP-β-CD complexes according to the following equation:

$$\alpha = \frac{50}{[G]_{tot}}\left([G]_{tot} - [CD]_{tot} - K_{(1:1)}^{-1}\right) + \left[\frac{50^2}{[G]_{tot}^2}\left([G]_{tot} - [CD]_{tot} - K_{(1:1)}^{-1}\right)^2 + 10^4/[G]_{tot} \times K_{(1:1)}\right]^{1/2}$$

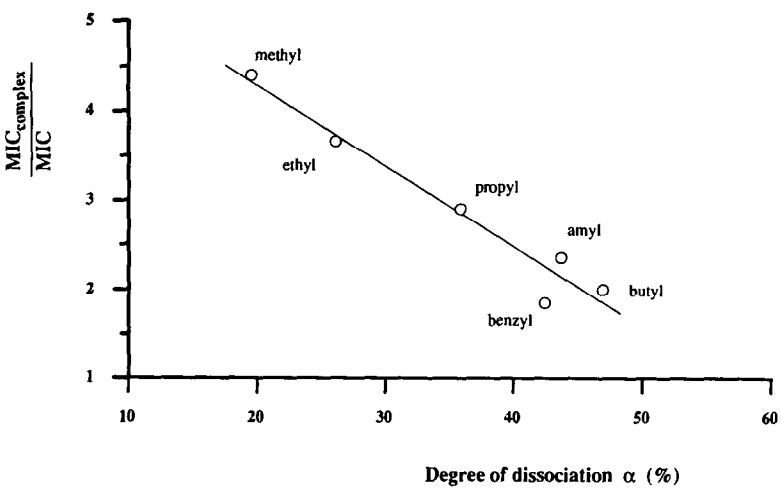

Fig. 7. Effect of dissociation of PHB ester-HP-β-CD-complexes on decrease of antimicrobial activity against *Candida albicans*.

where $[G]_{tot}$ is the molar concentration of PHB ester and $[CD]_{tot}$ the molar concentration of HP-β-CD.

Fig. 7 shows the very good linear correlation between dissociation of the complexes and inactivation of the series of the PHB esters (Eqn 7 in Table 2). It is obvious that the loss of antimicrobial activity depends only on the fraction of preservative which is included in the cyclodextrin molecule. Only the free part can be used for antimicrobial attack. Using this equation it is now possible to calculate the loss of activity when HP-β-CD is added to solutions preserved with any PHB ester. Only complex stability constants are required for such calculations and thereby allow the scale of biological experiments to be reduced.

Acknowledgement

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