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Influence of methanol and cyclodextrin cavity size on stoichiometry and binding constant of 3-[2-(9-anthryl)benzoxazol-5-yl]-alanine

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Abstract

A newly synthesized amino acid derivative 3-[2-(9-anthryl)benzoxazol-5-yl]-alanine was applied to study the influence of methanol concentration on equilibrium constant with β-cyclodextrin (β-CD) and γ-cyclodextrin (γ-CD). For both CDs the formation of ternary CD/guest/methanol complexes were established. The increase of methanol concentration decreases the apparent equilibrium constant for β-CD, whereas for γ-CD an increase was observed up to about 1 M of MeOH and then monotonical decrease with further increase of alcohol concentration. Calculated from the dependence of apparent equilibrium constant on methanol concentration, equilibrium constants of binary complex between β-CD and amino acid is equal to 780 M^{-1}, between β-CD and methanol 2 M^{-1} and 410 M^{-1} for ternary complex. The apparent thermodynamic parameters for complex formation of amino acid studied with β- and γ-CD in water/methanol mixture (2%, v/v) are: $\Delta S = -32.7 \pm 2.5$ J/mol K, $\Delta H = -25.3 \pm 0.7$ kJ/mol; $\Delta S = 24.9 \pm 3.3$ J/mol K, $\Delta H = -2.7 \pm 1.0$ kJ/mol for β-CD and γ-CD, respectively.

Keywords: Methanol; Cyclodextrin; Amino acid

1. Introduction

Cyclodextrins (CDs) are toroidally shaped polysaccharides made up of six to eight D-glucose monomers connected at the 1 and 4 carbon atoms. The cavities of CDs are relatively hydrophobic and have an internal diameter of 4.7–8.3 Å [1,2]. This difference in cavity size allows binding specificity to be tailored based on substrate size and geometry. Therefore, CDs are ideal molecules for the study of small molecule binding. There are many studies dealing with inclusion complexes of CDs [3–23]. The structure of a supramolecule consisting of CD as a host and organic molecule along with several solvent molecules as guests is quite well defined [24].

Imperfect fit of the guest to the CD cavity leads to association constant decrease. If the guest does not occupy the whole internal space of the cavity, the void space will be filled with solvent molecules. In the case of water, its incorporation is thermodynamically unfavourable. The efficiency of CD complexation can be altered by addition of ternary complexation agents, such as alcohols leading to either a decrease [3,4,7,16] or increase [15,21] of the CD–guest association constants. Enhancement of the complexation efficiency has been frequently attributed to the formation of ternary complexes, where the third complexation agent replaced the water molecules in the void space of the CD cavity. In contrast, when the equilibrium constants decrease the third complexation agent may partially displace the guest molecule within the CD. Recently, much attention has been focussed on the use of alcohols as the third component, since they could affect the formation of the original binary complex. Alcohols are the simplest organic analogues of water, which is known to be essential in inclusion process. The size and geometry of the alcohol seem to be important factors [25,26] as is its ability to alter the hydrophobicity of the cavity [27]. The presence of primary and secondary hydroxyl groups in the CD is also essential for the formation of the ternary alcohol complexes [26], whose stoichiometry depends on the specificity of the guest, the type of CD, and the type of alcohol [3,4,7,15,26,28].

It is worth noting that the alcohols themselves are able to form the complex with CD. The strength of the binding of

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alcohol depends on the number of the carbon atoms in the chain of the alcohol and the type of its alkyl chain (linear or branched) [3,4,7,15,28]. For short linear alcohols the equilibrium constants are rather low. The literature values of the binding constants to the β-CD cavity for methanol, propanol or pentanol are 0.32, 3.7 and 63 M^{-1}, respectively [28].

Although a large number of systems are capable to form inclusion complexes with CDs, the changes of the photophysical properties on encapsulation are in many cases too small to provide any meaningful and reliable information on the micro-heterogeneity of the CD or the location of the probe. This necessitates further studies of the complexation process involving probes whose photophysical properties are sensitive to any small change in their environment. In the present study, we are examining the effect of CD cavity size as well as the methanol concentration, the solvent usually used to dissolve bad water-soluble compounds [13,22], on the binding constant of newly synthesized amino acid derivative 3-[2-(9-anthryl)benzoxazol-5-yl]-L-alanine (Box-Ant) showing high sensitivity on polarity of its environment.

2. Material and methods

CDs were purchased from Roth and used without further purification, MeOH (Lab-Scan) was HPLC grade, cyclohexane (Merck) was for fluorescence spectroscopy, whereas water was deionized using a Millipore Milli-Q system. 3-Nitro-tyrosine (Tyr(NO_2)), *N*-bromosuccinimide (NBS), 9-anthraldehyde were from Lancaster and were used as received.

Box-Ant (Fig. 1) was synthesized as described below.

N-[(*tert*-Butoxy)carbonyl]-Box-Ant methyl ester was synthesized from N^{α}-Boc-protected 3-amino-L-tyrosine methyl ester and 9-anthraldehyde as substrates, via the intermediate Schiff base, which underwent oxidative cyclization to the heterocyclic compound in the presence of NBS, according to the procedure published previously [29]. The product was isolated from the reaction mixture by means of column chromatography (Macherey Nagel, silica gel 60, 0.063–0.2 mm) using as an eluent AcOEt/petroleum ether 1:2. Then it was purified by means of semi-preparative RP-HPLC (Kromasil column, C-8, 5 μm, 250 mm long, ID = 20 mm). The mobile phase was a gradient running from 40 to 80% of aqueous solution of acetonitrile with addition of 0.1% trifluoroacetic acid (TFA) over 60 min with detection at 250 nm. The total yield of the product was 8.9%. The purity of the obtained compound (yellow solid) was checked by means of TLC (Merck plates, Kieselgel 60 F_{254}, AcOEt/petroleum ether 1:2, $R_f = 0.73$) and analytical RP-HPLC (Kromasil column, C-18, 5 μm, 250 mm long, ID = 4.5 mm) with detection at $\lambda = 250$ nm. The mobile phase was a gradient running from 40 to 80% of aqueous solution of acetonitrile with addition of 0.1% TFA over 30 min plus acetonitrile over 10 min ($t_R = 30.9$ min). The identification of the product was based on the ^{1}H NMR spectrum recorded on Varian, Mercury-400 BB spectrometer (400 MHz) in $CDCl_3$, δ (ppm): 1.46 (9H, s, $(CH_3)_3$), 3.33 (2H, m, $C^{\beta}H_2$), 3.81 (3H, s, $-OCH_3$), 4.73 (1H, m, $C^{\alpha}H$), 5.16 (1H, d, $J = 8.0$ Hz, NH), 7.26 (1H, m, C^6H), 7.53 (4H, m, $C^{2'}H$, $C^{3'}H$, $C^{6'}H$, $C^{7'}H$), 7.64 (1H, d, $J = 8.4$ Hz, C^7H), 7.73 (1H, s, C^4H), 8.07 (4H, m, $C^{1'}H$, $C^{4'}H$, $C^{5'}H$, $C^{8'}H$), 8.68 (1H, s, $C^{10'}H$).

The methyl ester removal was performed using 1 N solution of NaOH in methanol. The Boc removal was performed using 6.8 M HCl in dioxan. The product was isolated by means of semi-preparative RP-HPLC (Supelcosil^{TM} SPLC-ABZ column, 5 μm, 250 mm long, ID = 10 mm). The mobile phase was a gradient running from 8 to 80% of aqueous solution of acetonitrile with addition of 0.1% TFA over 120 min with detection at $\lambda = 223$ nm. The total yield of Box-Ant was 20.7%. The purity of the obtained compound (yellow solid) was checked by means of analytical RP-HPLC (Kromasil column, C-18, 5 μm, 250 mm long, ID = 4.5 mm) with detection at 370 nm. The mobile phase was a gradient running from 8 to 80% of aqueous solution of acetonitrile with addition of 0.1% TFA over 30 min plus 80% of aqueous solution of acetonitrile over 30 min ($t_R = 20.7$ min).

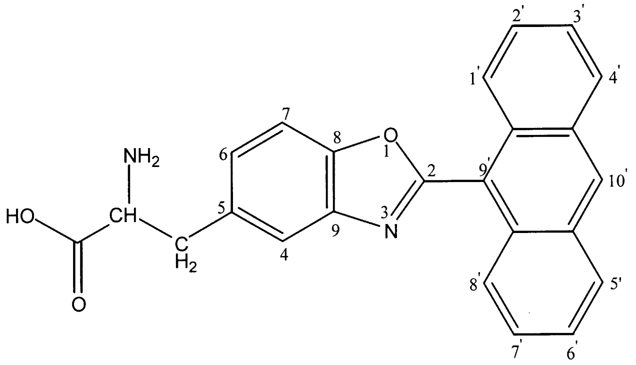

Fig. 1. Structure of 3-[2-(9-anthryl)benzoxazol-5-yl]-alanine.

2.1. Spectroscopy measurements

The solutions were prepared by dissolving the appropriate amount of CD in water. To a volume of 3.0 ml of aqueous solution of β-CD or γ-CD, 60 μl of methanolic solution of Box-Ant was added, so that the methanol concentration was 2% (v/v), while the Box-Ant final concentration was equal to 1.9×10^{-5} M. The optical density of the sample at the excitation wavelength ($\lambda = 384$ nm) did not exceed 0.1. Absorption spectra were recorded using a Perkin-Elmer Lambda 18 spectrophotometer.

Fluorescence spectra were recorded using a Perkin-Elmer LS-50B spectrofluorimeter with 2.5 nm bandwidth for excitation and emission. For the equilibrium constant calculations the steady-state emission spectra, the integral fluorescence intensity (corrected for absorbance) as well as the fluorescence intensity measured at 450, 500, 525, 550 and 590 nm were used. The steady-state emission spectra in pure solvents were performed at 25 °C, whereas in the case of mixture of Box-Ant and CDs at 10, 17, 25, 35 and 50 °C. Temperature was maintained using Julabo F26-MP refrigerated circulator.

In the studies of influence of methanol concentration on the equilibrium constant methanol was added into Box-Ant/CD mixture. The final concentration of MeOH was 2, 4, 6, 10 and 15% for β-CD and 1.67, 2, 3, 5, 7, 10 and 15% for γ-CD. All samples containing CD were held overnight to equilibrate.

Fluorescence quantum yields were measured relative to quinine bisulphate (QY = 0.53 [30]) at room temperature and were corrected for different refractive indexes of the solvents. Equilibrium constants calculations were performed using Origin 6.1 software from OriginLab, Northampton, MA.

2.2. Time-resolved fluorescence intensity decay measurements

Fluorescence decays were collected using a time-correlated single-photon counting apparatus (the picosecond/femtosecond laser system, Ti:sapphire 'Tsunami' laser pumped with an argon ion laser 'BeamLok' 2060 and thermoelectrically cooled MCP-PTM R3809U-05) at the Laboratory of Ultrafast Laser Spectroscopy, Adam Mickiewicz University, Poznań, Poland [31]. The emission wavelength (510 nm) was selected by means of monochromator (about 7.5 nm bandwidth) and was detected with a magic-angle polarizer. The fluorescence intensity decays were recorded at 20 °C with a polarizer set up at a magic-angle. The Ludox solution was used as a reference.

Fluorescence intensity decay data were fitted by the iterative convolution to the sum of exponents:

$$I(t) = \sum_{i}^{n} \alpha_i \exp\left(-\frac{t}{\tau_i}\right) \qquad (1)$$

where α_i and τ_i are the pre-exponential factor and fluorescence lifetime, respectively.

The adequacy of the exponential decay fitting was judged by visual inspection of the plots of weighted residuals and by the statistical parameter χ^2_R, and shape of the autocorrelation function of the weighted residual, and serial variance ratio (SVR).

3. Results and discussion

Absorption and fluorescence spectra of new, highly fluorescent amino acid derivative Box-Ant in non-polar (cyclohexane) and polar (methanol and water) solvents are presented in Fig. 2. In all solvents studied the longest absorption band of the absorption spectrum shows vibrational structure whose sharpness only slightly depends on the solvent polarity, whereas the molar absorption coefficient at maximum of the long-wave absorption band (λ = 384 nm) substantially decreases from cyclohexane (8670 dm^3 mol^{-1} cm^{-1}) to water (3600 dm^3 mol^{-1} cm^{-1}) (Table 1).

The fluorescence spectra in all solvents studied are structureless and their maxima are shifted to the longer wavelength with the increase of solvent polarity (Fig. 2). The fluorescence quantum yield substantially depends on the solvent polarity and is equal to 0.64 for cyclohexane, 0.22 for methanol and 0.017 for water (Table 1). Also, the char-

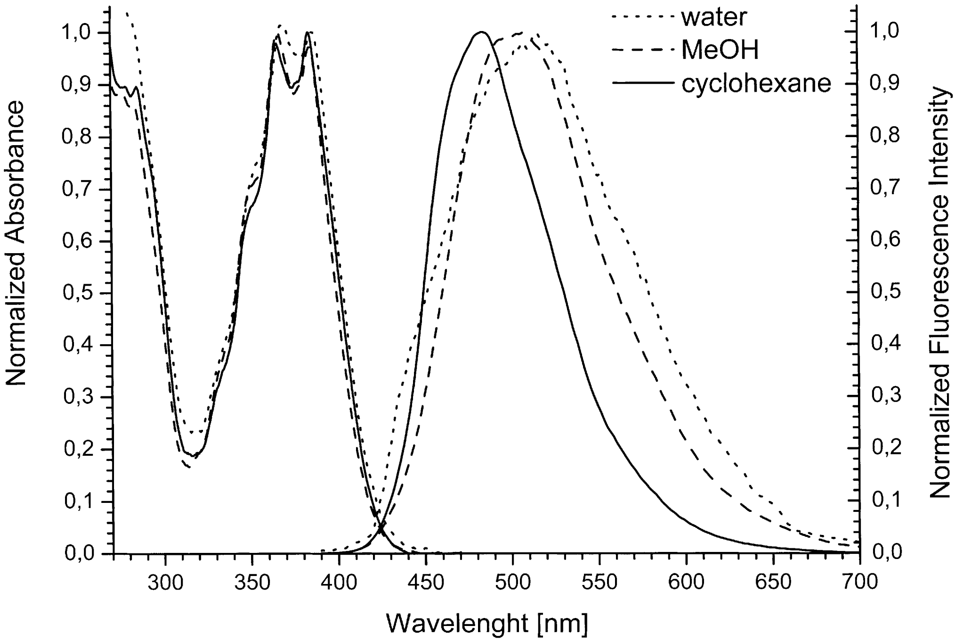

Fig. 2. Normalized absorption (left) and emission spectra (right) of 3-[2-(9-anthryl)benzoxazol-5-yl]-alanine in water, methanol and cyclohexane.

Table 1
Spectroscopic and photophysical properties of Box-Ant in cyclohexane, methanol, water and in water/CDs mixture

Solvent	ε (dm^3 mol^{-1} cm^{-1})	QY	τ_i (ns)	α_i	χ^2_{R}[a]
Cyclohexane	8668	0.637	5.46	1	0.99
Methanol	5178	0.223	3.75	1	1.16
Water	3599	0.02	0.53	1	14.03
			0.37	0.975	1.12
			5.18	0.025	
Water + 0.0154 M β-CD	4163	0.161	2.78	1	17
			3.94	0.271	1.68
			1.16	0.729	
			5.19	0.099	1.14
			2.44	0.408	
			0.65	0.493	
Water + 0.1684 M γ-CD	4519	0.151	2.76	1	39.5
			4.40	0.223	1.22
			1.71	0.777	
			5.55	0.083	1.03
			2.53	0.538	
			1.00	0.379	

[a] For 1, 2 or 3 exponential fit.

acter of fluorescence intensity decay as well as fluorescence lifetime of Box-Ant depends on the nature of a solvent used (Table 1). In cyclohexane, fluorescence intensity decay is mono-exponential and fluorescence lifetime is equal to 5.46 ns and longer compared to that of 9-methyl-anthracene in this solvent [32]. In methanol mono-exponential fluorescence intensity decay is also observed but fluorescence lifetime is much shorter (3.75 ns) than in cyclohexane and shorter than that of 9-methyl-anthracene in methanol. In water, double-exponential function is needed to fit adequately to the fluorescence intensity decay of Box-Ant (Table 1). The fluorescence lifetimes are equal to $\tau_1 = 0.37$ ns and $\tau_2 = 5.18$ ns and pre-exponential factors $\alpha_1 = 0.975$ and $\alpha_2 = 0.025$. Aggregates forming by highly hydrophobic Box-Ant seem to be responsible for heterogeneous fluorescence decay in water. The small contribution of long-lifetime component approximately equal to the fluorescence lifetime in cyclohexane supports such suggestion. The real concentration of such aggregate in solution is much lower than its contribution to the fluorescence intensity decay (2.5%) because of much higher fluorescence quantum yield of Box-Ant in non-polar solvent than in water.

3.1. Complex formation constants

Increase of the concentration of the β- or γ-CD in solution containing 3-[2-(9-anthryl)benzoxazol-5-yl]-alanine caused an increase of the Box-Ant fluorescence intensity and simultaneously changed the shape of the emission band (Fig. 3). The plots of the integral intensity (area under fluorescence band) corrected for absorbance change versus the β-CD concentration are shown in Fig. 4. One can use this intensity increase to determine the ground-state association constant of Box-Ant with the β-CD. For a 1:1 complex the association process

$$\text{Box-Ant} + \beta\text{-CD} \rightleftarrows \text{Box-Ant} : \beta\text{-CD} \qquad (2)$$

has the association constant defined by

$$K_1 = \frac{[\text{Box-Ant} : \beta\text{-CD}]}{[\text{Box-Ant}][\beta\text{-CD}]} \qquad (3)$$

Because of the low solubility of Box-Ant in water, the compound studied was dissolved in methanol and added to the water solution of CDs, this is a well-know procedure implemented in such cases [13,22]. The final methanol concentration in a mixture was 2% (v/v). In the systems containing alcohol the following two equilibria must also be considered:

$$\text{ROH} + \beta\text{-CD} \rightleftarrows \text{ROH} : \beta\text{-CD} \qquad (4)$$

$$\text{Box-Ant} + \text{ROH} : \beta\text{-CD} \rightleftarrows \text{ROH} : \text{Box-Ant} : \beta\text{-CD} \qquad (5)$$

These two equilibria together with Eq. (2) can be combined into the general process:

$$\text{Box-Ant} + \beta\text{-CD} + \text{ROH} \rightleftarrows (\text{Box-Ant})_{\text{bound}} \qquad (6)$$

for which the apparent association constant is

$$K_{\text{app}} = \frac{[(\text{Box-Ant})_{\text{bound}}]}{[\text{Box-Ant}][\beta\text{-CD}]} \qquad (7)$$

The procedure for extracting the value of K_{app} from the binding isotherm data has been described in many papers [3,4,9,12,15,16,18,33]. The 1:1 and 1:2 binding constants can be determined using the Benesi–Hildebrand equation from double reciprocal plots as given by the following equations:

$$\frac{1}{I - I_0} = \frac{1}{(I_\infty - I_0)K_{\text{app}}[\text{CD}]} + \frac{1}{I_\infty - I_0} \qquad (8)$$

$$\frac{1}{I - I_0} = \frac{1}{(I_\infty - I_0)K_{\text{app}}[\text{CD}]^2} + \frac{1}{I_\infty - I_0} \qquad (9)$$

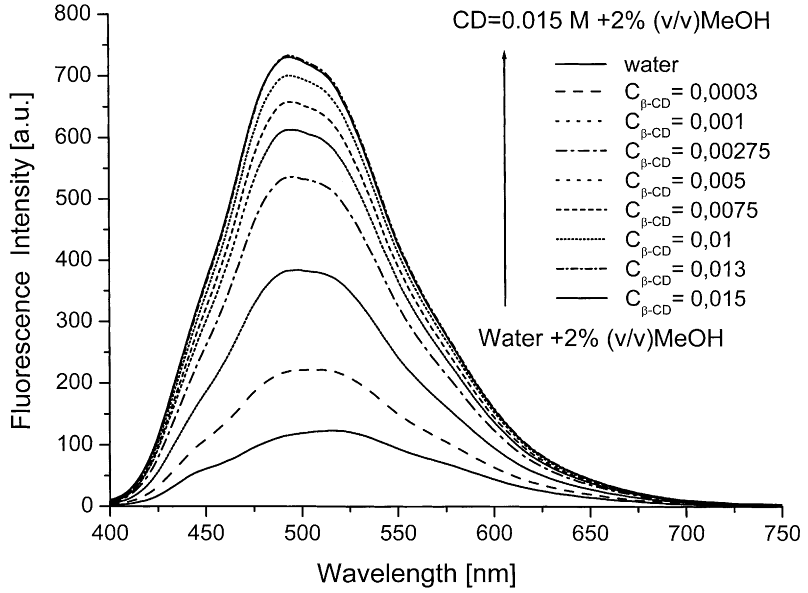

Fig. 3. Fluorescence spectra of 3-[2-(9-anthryl)benzoxazol-5-yl]-alanine recorded at different β-CD concentrations.

where I_0 and I_∞ denote the fluorescence intensity of guest, in the absence and in the presence of the CD excess, respectively, [CD] represents host concentration and K_{app} the apparent binding constant. For a 1:1 host–guest complex, plot of $1/(I_0 - I_\infty)$ versus [CD] should be linear, and a plot of $1/(I_0 - I_\infty)$ versus $[\mathrm{CD}]^2$ assuming a 2:1 association should reveal a line.

Unfortunately, Benesi–Hildebrand plots tend to place more emphasis on lower concentration values than on higher ones. Consequently, the slope of the line is more sensitive to the ordinate value of the point representing the smallest concentration. Thus, in order to accurately weight the data, it is necessary to employ a non-linear regression package and fit the experimental data directly according to the equation [4,9,12,18,33,34]

$$I = \frac{I_0 + I_\infty K_{\mathrm{app}}[\mathrm{CD}]}{1 + K_{\mathrm{app}}[\mathrm{CD}]} \tag{10}$$

The integral emission was treated according to Eqs. (8) and (10) (Fig. 4). The excellent linear double reciprocal

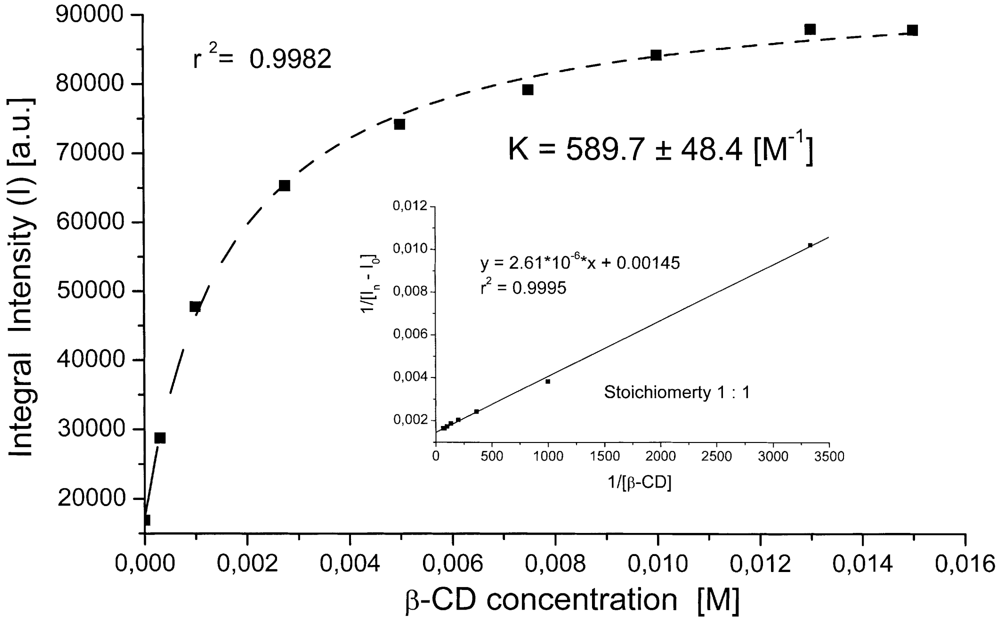

Fig. 4. Dependence of integral intensity on β-CD concentration. The dashed line represents the best fit of experimental data to Eq. (10). Inset shows the double reciprocal plot and the best fit of experimental data to Eq. (8) (solid line).

plot ($r^2 = 0.9995$) as well as excellent fit to Eq. (10) ($r^2 = 0.9982$) indicate that a 1:1 complex between β-CD and Box-Ant is forming with K_{app} equal to $590 \pm 48\,M^{-1}$. Apart from integral intensity (quantum yield) other photophysical properties proportional to the concentration of the free and bound guest molecule can be used for stoichiometry and binding constant calculation. As we state in our previous paper [18] fluorescence intensity or normalized pre-exponential factors (contribution) of the fluorescence lifetime of all forms presented in solution obtained from the global analysis of the fluorescence intensity as well as the average fluorescence lifetimes can be used for such calculations. The fluorescence decays of Box-Ant in water solution with and without CDs are highly heterogeneous (see Table 1) so, it is impossible to assign particular lifetime to a particular form of Box-Ant (free or complexed with CD) present in solution. Thus, normalized pre-exponential factors are excluded from calculations of stoichiometry and binding constant value. The uncertainties of the equilibrium constant determined using photophysical properties depend on the degree of their change after complex formation. For larger changes of appropriate parameters the uncertainties in the equilibrium constant are adequately smaller [18]. The ratio of average fluorescence lifetimes of Box-Ant bound to the CDs cavity to the free form is 3.7 for β-CD and 4.5 for γ-CD, which is lower than the ratio of fluorescence integral intensity (about 5) or fluorescence intensity at chosen wavelength (about 6). Therefore, we decided to base our calculation of the binding constant on the steady-state data. As can be seen from Fig. 2 the shape of the fluorescence band is changing and the band becomes blue shifted with CDs concentration increase. In order to check whether these changes are caused by forming complexes with different stoichiometry or polarity of micro-environment of Box-Ant, the stoichiometry and equilibrium constant were computed using a fluorescence intensity at five different wavelengths chosen across the fluorescence band (450, 500, 525, 550 or 590 nm). The same character of the plot as presented in Fig. 4 was obtained (figure not shown) indicating that the observed changes are caused by polarity changes. Because, simultaneous analysis of a several sets of data (global analysis) gives more accurate value of calculating quantity, all binding constant calculation are determined based on global analysis using fluorescence intensity at the aforementioned wavelengths.

The K_{app} values can be used to obtain the binding constants of the alcohol to CDs if they are estimated at different alcohol concentrations. In principle three equilibria must be considered, each described by its own equilibrium constant: K_1 (Eq. (2)), K_2 (Eq. (4)) and K_3 (Eq. (5)). The relation between K_{app} and these three equilibrium constants is given by [4,34]

$$K_{app} = \frac{K_1 + K_2 K_3[\mathrm{MeOH}]}{1 + K_2[\mathrm{MeOH}]} \tag{11}$$

Table 2
Influence of MeOH concentration on binding constant of Box-Ant with β-CD and γ-CD

MeOH		K_{app} (M^{-1})	
% (v/v)	c (M)	β-CD	γ-CD
1.67	0.412	–	50 ± 11
2	0.485	591 ± 19	62 ± 4
3	0.742	–	86 ± 5
4	0.99	527 ± 8	–
5	1.236	–	92 ± 8
6	1.485	501 ± 13	–
10	2.467	472 ± 8	46 ± 6
15	3.708	448 ± 22	27 ± 7

In Table 2 and Fig. 5 the dependence of K_{app} values as a function of the methanol concentration for β-CD and γ-CD are shown. The addition of methanol had an opposite effect on the ground state Box-Ant complex formation with β- and γ-CD. For β-CD-Box-Ant system the addition of methanol leads to the decrease of the apparent binding constant in all range of methanol concentration studied, whereas for γ-CD-Box-Ant system an increase of K_{app} was observed when the methanol concentration increased to about 1 M and then monotonous decrease for further MeOH concentration increase. This can be attributed to different size of the cavity of the CDs studied. The smaller cavity of β-CD has less space to accommodate Box-Ant and the alcohol, resulting in less favourable Box-Ant complexation. However, in the larger cavity of γ-CD there is enough space to incorporate the alcohol molecules. The decrease of K_{app} with an increase of the alcohol concentration for β-CD suggests a weaker interaction of Box-Ant with CD in the presence of alcohol. There were two possible reasons of this observation exclusion of Box-Ant from CD cavity, due to occupation of the internal space by alcohol molecules as observe by van Stan et al. [4] for 2-naphthol complex or ternary complex formation between CD, alcohol and Box-Ant. In the latter case, the smaller change of fluorescence intensity in the presence of methanol suggested a smaller equilibrium constant for the ternary complex compared to the Box-Ant-CD equilibrium ($K_2 < K_1$) as observed by Liao and Bohne [34] for xanthon and Schuette et al. [3] for acridine complex with β-CD. To resolve this issue, K_{app} values were measured for different MeOH concentration and fitted to Eq. (11). The value of K_{app} for which the equilibrium constants for the ternary complexes were determined, incorporated the term for alcohol concentration. This was a valid approach since the alcohol concentration in all cases was much higher than the CD concentration. For this treatment, we cannot establish whether one or more alcohol molecule were involved in the complexation.

The results obtained from the fit of the K_{app} to Eq. (11), in which all equilibrium constants were determinable parameters, for β-CD/Box-Ant complex formation gave the following equilibrium constants: $K_1 = 780 \pm 65$, $K_2 = 2.1 \pm 0.8$

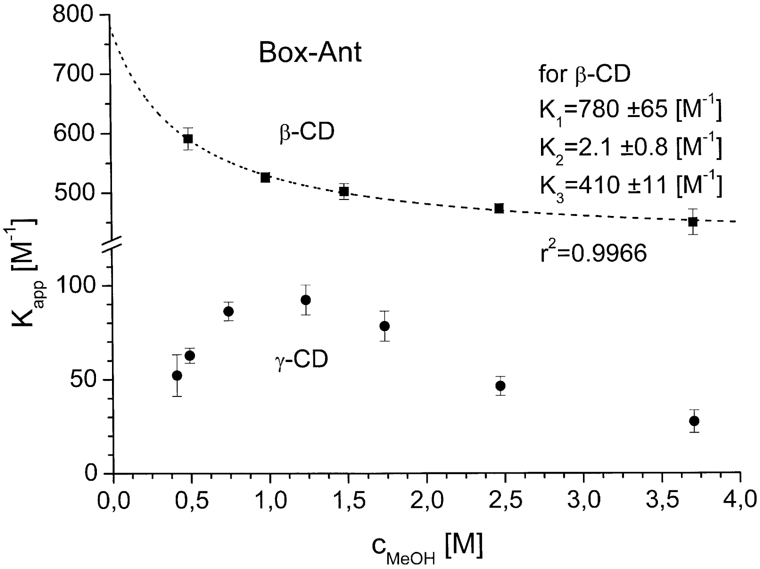

Fig. 5. Dependence of apparent equilibrium constants on methanol concentration at constant β- and γ-CD concentration. The dashed line represents the best fit of K_{app} to Eq. (11).

and $K_3 = 409 \pm 11$ ($r^2 = 0.9966$). The equilibrium constant for binary β-CD/Box-Ant complex formation in accordance with Eq. (2) is equal to $K_1 = 780\,M^{-1}$ and is higher than equilibrium constant of ternary complex formation $K_3 = 409\,M^{-1}$ (Eq. (5)). The equilibrium constant K_2 describes the binding strength of methanol to β-CD (Eq. (4)) and it is in good agreement with the value $0.4\,M^{-1}$ obtained by Hami [28] and is much lower than those of binary and ternary complex formation. Ternary complexation agents, such as alcohols, were suggested to replace water molecule for the solvation of the empty CD cavity and/or CD–guest complex. If the solvation of empty cavity by the alcohol molecules are strong, release of the guest molecule into the aqueous phase will be observed with increasing concentration of alcohol. However, ternary complex CD/alcohol/guest will be formed when the solvation of the CD with an alcohol does not lead to the formation of strong inclusion complexes but is thermodynamically favourable than the solvation by water molecule. Thermodynamically unfavourable hydration of the bulky aromatic group of Box-Ant should be manifested as high enthalpy of complex formation with CDs. The thermodynamic parameters of the complex formation between Box-Ant and β- and γ-CD in methanol/water mixture (2%, v/v) obtained from van't Hoff equation are presented in Table 3. High enthalpy values as well as low entropy term obtained for β-CD (−25.3 and −32.7 J/mol K, respectively) indicate ternary complex formation in which methanol molecule fill up the void space inside β-CD replacing the water molecules. The decrease of the apparent equilibrium constant with the increase of methanol concentration is caused by steric effect rather than exclusion of Box-Ant molecule into the aqueous phase (relatively high K_3 equilibrium constant).

The addition of the methanol into the solution of Box-Ant in the presence of γ-CD led to an increase of K_{app} indicated that for γ-CD, which has larger cavity than β-CD, alcohol molecules were easily accommodated with Box-Ant inside the CD cavity. Thus, in the presence of alcohol, stronger complexes were formed, in absolute terms, with γ-CD than β-CD, reverting the pattern observed for binary complexes where the equilibrium constants are higher for the β-CD. The role of methanol as a ternary complexation agent was to fill up the void space inside or replaced water molecules within γ-CD cavity containing guest molecule which is

Table 3
Influence of temperature on binding constant of Box-Ant with β-CD and γ-CD in water/MeOH (2%, v/v) mixture

Temperature (K)	β-CD			γ-CD		
	K_{app} (M^{-1})	ΔS (J/mol K)	ΔH (kJ/mol)	K_{app} (M^{-1})	ΔS (J/mol K)	ΔH (kJ/mol)
283	937.8 ± 17.1	−32.7 ± 2.5	−25.3 ± 0.7	64.5 ± 3.3	24.9 ± 3.3	−2.7 ± 1.0
290	663.4 ± 31.6			–		
298	593.7 ± 20.6			62.5 ± 4.2		
308	398.0 ± 14.4			59.5 ± 3.6		
323	241.2 ± 9.2			52.8 ± 4.1		

thermodynamically favourable. To explain the monotonic decrease of K_{app} with further increase of MeOH concentration, in addition to the steric effect the role of hydrophobic interaction [34], specifically the interaction of Box-Ant with alcohol in homogeneous solution should also be considered. Methanol (6–15%) in water decreases the apparent equilibrium constant of guest molecule with γ-CD. For higher alcohol concentration in the water–methanol mixture the interaction of hydrophobic part of Box-Ant with methanol molecules as well as the hydrophilic amino acid moiety with water molecules causes that Box-Ant might be better solubilized in the homogeneous solution as the mole fraction of the cosolvent increased. Small energy of interaction of Box-Ant with γ-CD ($\Delta S = 24.9 \pm 3.3\,\mathrm{J/mol\,K}$, $\Delta H = -2.7 \pm 1.0\,\mathrm{kJ/mol}$), much smaller than with β-CD ($\Delta S = -32.7 \pm 2.5\,\mathrm{J/mol\,K}$, $\Delta H = -25.3 \pm 0.7\,\mathrm{kJ/mol}$), afforded that better solubility of Box-Ant in homogeneous solution plays a substantial role for γ-CD, decreasing K_{app}, whereas for β-CD this effect is not observed.

In summary, the Box-Ant forms stable ground-state complexes with both β-CD and γ-CD. The addition of methanol decreases the equilibrium constants of binary complex with β-CD and forms simultaneously stable ternary complex in all range of alcohol concentration studied. For γ-CD higher alcohol concentration increases stability of ternary complex, but further increase of methanol concentration causes substantial decrease of apparent equilibrium constant as a result of better solubility of Box-Ant in water–methanol homogeneous mixture and lower energy of interactions with γ-CD than with β-CD. These studies show that addition of co-including molecules can be employed to fine-tune of the host–guest complexation.

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