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Dimeric Cyclodextrin Carriers with High Binding Affinity to Porphyrinoid Photosensitizers

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Abstract. The aim of our investigation was to develop carrier systems for an application of inert drugs in photodynamic cancer therapy. β-Cyclodextrin dimers linked at their primary and secondary faces by spacers of varying lengths were synthesized as carrier systems. The binding constants of the inclusion complexes of these cyclodextrin dimers and porphyrinoid photosensitizers were determined by competitive spectrofluorometry. Particularly the secondary face linked dimers exhibited extremely high binding constants with values of 10^6–10^7 L/mol. Theoretical studies were carried out on these inclusion complexes to confirm the influence of spacer length and connecting side on complex stability.

Key words: Cyclodextrin dimers, inclusion complexes, binding constants, photodynamic therapy of cancer.

1. Introduction

One major drawback in photodynamic therapy (PDT) is the low selectivity of porphyrinoid photosensitizers towards tumor tissue. Porphyrinoid photosensitizers do not stain tumor tissue exclusively. Skin, eye retina and particularly liver, kidney, spleen, lung and blood cells are targets of these lipophilic drugs [1]. Only a combination of porphyrinoid photosensitizers with specific antibodies, creating a new class of drugs, might fulfill the desired specificity of photodynamic therapy, since one cannot expect a porphyrinoid molecule to have excellent photophysical properties on the one hand (absorption of far red light with high efficiency, optimum $^1O_2$ yield, etc.) and exclusive tumor specificity on the other hand.

To achieve this new class of drugs, the first prerequisite is a stable inclusion complex of a cyclodextrin dimer and a hydrophobic porphyrinoid photosensitizer, which would prevent photosensitizer transport on the lipoprotein pathway [2]. This would avoid any unwanted targeting of organs apart from the tumor, provided that

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a bond between the encapsulated drug and the tumor specific antibodies can be established.

Previous studies have shown that cyclodextrin dimers which possess suitable structures will bind guest molecules with high affinity [3–7]. Recently, a paper was published describing the synthesis of a β-cyclodextrin tetramer [8], a compound which represents a new approach to water-soluble complexing agents exhibiting very high affinity for tetraarylporphyrins with ionic substituents, with binding constants of 10^8 L/mol which exceeds the binding constant of cyclodextrin dimers. Inclusion complexes of β-cyclodextrin dimers with porphyrins have already been described in the literature. These complexes were used in supramolecular chemistry, molecular recognition and drug delivery [9, 10].

Monomeric cyclodextrins are widely used in various pharmaceutical applications [11], because of their ability to encapsulate hydrophobic guest molecules. In contrast to their use only as water-soluble encapsulation agents, we used cyclodextrin dimers as inert carriers in PDT to shield the encapsulated porphyrinoid photosensitizer from the surrounding lipoproteins. We investigated various primary and secondary face linked β-cyclodextrin dimers with regard to their binding ability to porphyrinoid photosensitizers.

2. Experimental

2.1. CHEMICALS

β-Cyclodextrin and heptakis(2,6-di-O-methyl)-β-cyclodextrin (Me-β-CD) were purchased from Wacker Chemie (Burghausen, Germany) and dried overnight at 120 °C. N,N'-Dimethylformamide (DMF) and pyridine were dried over calcium hydride and distilled before use. 6-(p-Toluidino)-2-naphthalenesulfonic acid (TNS) was obtained from Aldrich and used without further purification. All other chemicals were analytical grade reagents. Thin-layer chromatography was performed on HPTLC plates obtained from Merck (Silica Gel 60 F 254), eluants were as follows: A = butanol/ethanol/water 5 : 4 : 3, B = 2-propanol/water/ethylacetate/ammonium hydroxide (25%) 10 : 8 : 6 : 1. TLC spots were visualized with a spray reagent consisting of a stock solution of 0.1 g anthrone in 100 mL sulfuric acid, diluted 1 : 100 with ethanol, followed by heating to 80 °C. Column chromatography was performed on LiChroprep RP-8 silica gel obtained from Merck and on Q-Sepharose and DEAE-Sephadex obtained from Pharmacia (Uppsala, Sweden). Mass spectra were obtained by matrix assisted laser desorption ionisation (MALDI) on a non-commercial linear time-of-flight spectrometer. Post source decay (PSD)-MALDI-MS spectra were recorded on a modified LAMMA 1000 reflection time-of-flight spectrometer. The matrix used was 2,5-dihydroxy-benzoic acid [12]. Fluorescence spectra were recorded on a Perkin Elmer LS 50.

2.2. PSD-MALDI-MS

The PSD-MALDI technique was described in detail earlier [13, 14]. This method is based on the mass analysis of fragment ions formed by unimolecular or collision induced decay [15]. This decay takes place in the first field free drift path of a reflectron time-of-flight mass spectrometer. The molecule ions were accelerated to a kinetic energy of 10 keV. Samples were prepared using 2,5-dihydroxybenzoic acid (DHB) as a matrix. Typically, 1–10 pmol analyte was used and mixed with a DHB-solution (10 mg/mL in ethanol/water 1 : 1 v/v) up to a molar ratio of 1 : 1000.

2.3. FLUORESCENCE MEASUREMENTS

Fluorescence measurements were performed in a 0.1 M sodium hydrogen carbonate buffer (adjusted to pH 9 with sodium hydroxide) using a Perkin Elmer LS 50 spectrofluorometer. The excitation wavelength was 320 nm. The fluorescence emission was monitored at 460 nm for Me-β-CD, 448 nm for 6,6′S-cyclodextrin dimers and 419–428 nm for 2,2′N-cyclodextrin dimers.

2.4. DETERMINATION OF BINDING CONSTANTS

Binding constants of TNS with Me-β-CD and CD dimers were determined from the dependence of TNS fluorescence intensity on the concentration of cyclodextrin as described elsewhere [16, 17]. The fluorescence intensity enhancement of TNS (1.0×10^{-5} M^{-1}) after addition of increasing amounts of cyclodextrin dimers or Me-β-CD was observed at the emission maximum. Cyclodextrin concentrations were chosen in a range of complexation between 20% and 80% (5.0×10^{-5}–3.0×10^{-4} M^{-1} for dimers, 1.0×10^{-4}–1.0×10^{-3} M^{-1} for Me-β-CD). At the first run TNS as guest was present. The second run followed the same procedure with an addition of a fixed concentration of the corresponding porphyrinoid derivative as a competitor. This concentration was chosen to be as low as possible to detect any competition. All experiments were performed in triplicate. The binding constants of β-cyclodextrin dimers with porphyrinoid derivative were determined in this indirect method by monitoring the attenuation of the TNS fluorescence intensity [17]. Double reciprocal plots of the titration data from the first run (with guest TNS) and from the second run (with competitive guest) gave straight lines (Figure 5). The binding constants of β-cyclodextrin dimers with TNS were calculated from the ratio of the y-intercepts to the slopes of the first run's curve, as shown in Figure 5. The binding constants of β-cyclodextrin dimers with guest molecules can be calculated by use of the equilibria of competitive inhibition [18, 19]. The effect of the competitive inhibitor is to produce an apparent increase in K_2 by the

factor $(1 + [\mathrm{I}]/K_i)$, which grows larger as [I] increases. Therefore, the dissociation constant K_i can be formulated as:

$$\frac{[\mathrm{I}]}{\left(\frac{K_1}{K_2} - 1\right)} = K_i$$

where K_i = dissociation constant for the guest, $1/K_i$ = binding constant; K_1 = negative x-intercept of first run curve (binding constant for TNS); K_2 = negative x-intercept of second run curve; [I] = competitive guest concentration.

The binding constant is defined as $1/K_{\text{dissociation}}$.

2.5. SYNTHESIS

Mono-2-tolylsulfonyl-2-deoxy-β-cyclodextrin, **3** was synthesized according to the literature method [20]. Purification was achieved by using reversed-phase column chromatography [21]. The column was eluted with water, followed by 5% ethanol, 10% ethanol, 15% ethanol and 20% ethanol. The 15% ethanol eluate contains the pure mono-2-tolylsulfonyl-2-deoxy-β-cyclodextrin. Evaporation and subsequent lyophilization yielded a white solid. Purity was checked by TLC using solvent A, R_{f} = 0.54, MALDI-MS $[\mathrm{M} + \mathrm{Na}]^+$ 1313.

Mono-6-tolylsulfonyl-6-deoxy-β-cyclodextrin, **2** and *mono-6-iodo-6-deoxy-β-cyclodextrin*, **4** were synthesized according to literature methods [22, 23].

Mono-6-(2-aminoethylthio)-6-deoxy-β-cyclodextrin, **6**. 2-Amino-ethanthiol (1.2 g, 16 mmol) and potassium carbonate (3.0 g, 22 mmol) were dissolved in 60 mL of anhydrous DMF. While the mixture was stirred at 70 °C a solution of mono-6-iodo-6-deoxy-β-cyclodextrin **4** (2.0 g, 1.6 mmol) dissolved in 40 mL of anhydrous DMF was added dropwise. After 3 h of stirring at 70 °C the potassium carbonate was filtered off and the filtrate was evaporated to a low volume. Acetone was added, producing a white precipitate. Recovery by filtration and drying *in vacuo* yielded the pure product (1.8 g, 94%); TLC (solvent B) R_{f} = 0.13; MALDI-MS $[\mathrm{M} + \mathrm{H}]^+$ 1195.

Alkylation of linear α,ω-diaminoalkanes with mono-2-tolylsulfonyl-2-deoxy-β-cyclodextrins. The following procedure applies to the synthesis of all mono-substituted ω-aminoalkylamino-β-cyclodextrins **5a**, **b**.

Mono-2-(ω-aminopropylamino)-2-deoxy-β-cyclodextrin, **5a**. l,3-Diamino-propane (2.5 mL, 30 mmol) was dissolved in 10 mL of water. Mono-2-tolylsulfonyl-2-deoxy-β-cyclodextrin **3** (4.0 g, 3 mmol) dissolved in 150 mL of water was added dropwise. After 3 h of stirring at 70 °C the solution was evaporated to a low volume. A mixture of acetone/methanol (40 : 1) was added, producing a white precipitate.

Recovery by filtration and drying *in vacuo* yielded the pure product (3.3 g, 93%); TLC (solvent B) R_f = 0.15; MALDI-MS $[M + H]^+$ 1192.

Synthesis of N-hydroxysuccinimide di-esters, **9a-d**. These well known compounds were prepared from the corresponding dicarboxylic acids and N-hydroxysuccinimide using 1,1′-carbonyldiimidazole as dehydrating reagent and DMF as a solvent.

Synthesis of 2,2′N-β-cyclodextrin dimers, **7a-d**. The following procedure applies to the synthesis of all 2,2′N-β-cyclodextrin dimers.

7b. Mono-2-(ω-aminopropylamino)-2-deoxy-β-cyclodextrin (3 g, 2.5 mmol) and di-succinimidyladipinate (0.5 g, 1.3 mmol) were dissolved in 60 mL of pyridine. The solution was heated for 2 h under reflux. After evaporation to a low volume acetone was added, producing a white-brownish precipitate. The precipitate was filtered off and was dissolved in water (adjusted to pH 8.0 with dilute sodium hydroxide) for further purification, using ion exchange chromatography (Q-Sepharose). The column was eluted with water, followed by a linear gradient ranging from water to 1 mol formic acid. The first cyclodextrin fraction contains the pure dimer. Evaporation and subsequent lyophilization yielded a white solid (2.2 g, 71%); TLC (solvent B) R_f = 0.06; MALDI-MS $[M + H]^+$ 2492.

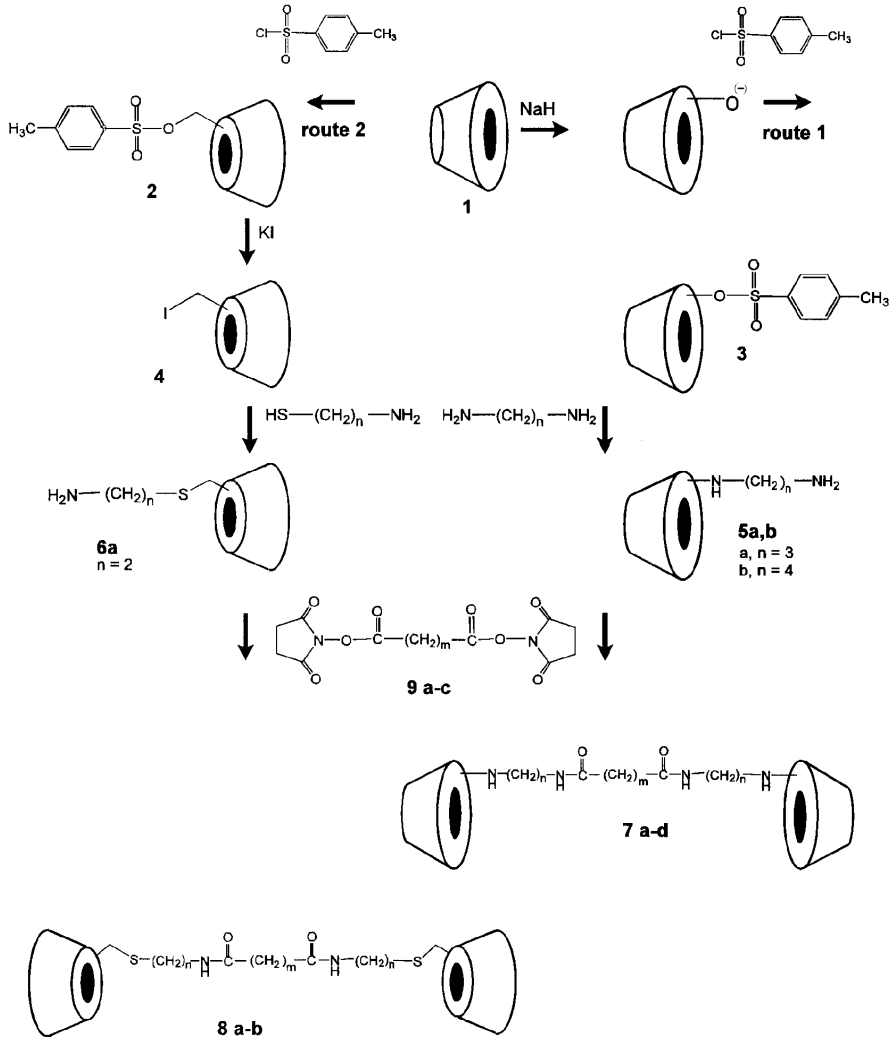
*Synthesis of 6,6′*S*-β-cyclodextrin dimers*, **8a-b**. The following procedure applies to the synthesis of all 6,6′S-β-cyclodextrin dimers.

8b. The synthesis was carried out as described for **7b** by using mono-6-(2-aminoethylthio)-6-deoxy-β-cyclodextrin **6** instead of mono-2-(ω-aminopropylamino)-2-deoxy-β-cyclodextrin. The purification of the crude cyclodextrin dimer was achieved by using ion exchange chromatography (DEAE-Sephadex). The column was eluted with water, followed by a linear gradient ranging from water to 0.5 mol formic acid. The first cyclodextrin fraction contains the pure dimer. Evaporation and subsequent lyophilization yielded a white solid (75%); TLC (solvent B) R_f = 0.07; MALDI-MS $[M + H]^+$ 2499.

3. Results

3.1. SYNTHESIS

Various primary and secondary face linked cyclodextrin dimers were successfully prepared by a multistep procedure, starting from monosubstituted β-cyclodextrin (Scheme 1). Mono-2-tolylsulfonyl-2-deoxy-β-cyclodextrin **3** was synthesized as previously described [20] and was purified using column chromatography [21]. After alkylation of linear α,ω-diaminoalkanes with mono-2-tolylsulfonyl-2-deoxy-β-cyclodextrin **3** (route 1) the resulting ω-aminoalkylamino substituted β-cyclodextrins **5a**, **b** were treated with N-hydroxysuccinimide esters **9a-c** to produce 2,2′N-β-cyclodextrin dimers [5]. Purification of the crude product by ion exchange chromatography yielded the desired dimers **7a-d** as shown in Figure 1. Side prod-



Scheme 1. Synthesis of β-cyclodextrin dimers.

ucts which eluted last, having only one β-cyclodextrin bound to the spacer (marked with arrows in Figure 1), were isolated, activated with N-hydroxysuccinimide and

Table I. β-cyclodextrin dimers and spacers.

Secondary face linked dimers		Primary face linked dimers		Spacer	
7a	$n = 3$, $m = 2$	**8a**	$n = 2$, $m = 3$	**9a**	$m = 2$
7b	$n = 3$, $m = 4$	**8b**	$n = 2$, $m = 4$	**9b**	$m = 3$
7c	$n = 4$, $m = 3$			**9c**	$m = 4$
7d	$n = 4$, $m = 4$				

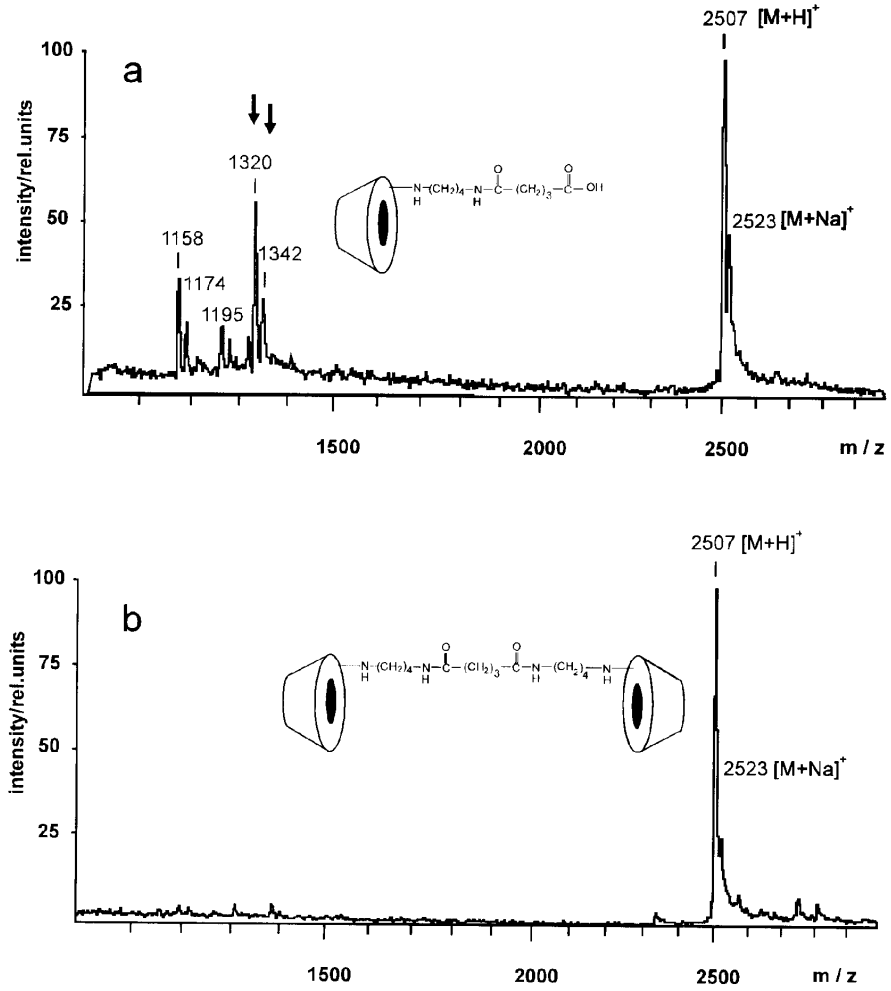


Figure 1. MALDI-MS of dimer **7c**. (a) (crude product) ↓ side products with only one β-cyclodextrin bound to the spacer [M + H] = 1320, [M + Na] = 1342, (b) after purification by ion exchange chromatography.

allowed to react with **5a** or **5b** respectively to produce the desired dimers. The analogous procedure of synthesizing 6,6′S-β-cyclodextrin dimers was not successful, probably due to the enhanced reactivity at the 6-position of the cyclodextrin. This multistep procedure terminated after the second step (6-ω-aminoalkylamino substituted β-cyclodextrin). However, this compound was a suitable tool to distinguish between a substitution at position 6 and position 2 of β-cyclodextrins using post-source decay matrix assisted laser desorption ionisation mass spectrometry (PSD-MALDI-MS) as described later.

A procedure starting from mono-6-iodo-6-deoxy-β-cyclodextrin **4** (route 2), as suggested by Breslow [7], followed by reaction with 2-amino-ethanthiol resulted in mono-6-(2-aminoethylthio)-6-deoxy-β-cyclodextrin **6**. 6,6′S-β-Cyclodextrin dimers were obtained by reaction of compound **6** with N-hydroxysuccinimide esters **9a-c**. Purification by ion exchange chromatography yielded the desired dimers **8a-b**. All synthesized β-cyclodextrin dimers are summarized in Table I.

3.2. PSD-MALDI-MS

Post-source decay matrix-assisted laser desorption ionisation mass spectrometry (PSD-MALDI-MS) [24] was used to characterize the synthesized cyclodextrin derivatives. The strong tendency of laser-desorbed carbohydrate ions to decay on a long time scale during flight through the mass spectrometer can be used to determine the structure of substituted cyclodextrins. Several characteristic bond cleavages observed in PSD-MALDI allow us to distinguish linkage positions and modification sites [12].

PSD spectra of mono-2-(ω-aminobutylamino)-2-deoxy-β-cyclodextrin **5b** and mono-6-(ω-aminobutylamino)-6-deoxy-β-cyclodextrin are shown in Figure 2. These structurally isomeric compounds were chosen in order to confirm the position of derivatization in the cyclodextrin monomer and, hence, in the resulting dimers **7a-d**. The spectra are characterized by a high reproducibility of the fragmentation pattern and the signal intensities and by a very good signal-to-noise ratio even at low sample concentrations.

For all signals observed, a glycosidic bond cleavage is assumed as the first step of fragmentation, opening the cyclodextrin ring, followed by one or more further fragmentations that lead to the observed ion signals. It is a characteristic feature of PSD-MALDI of carbohydrates, that multiple consecutive fragmentation reactions can take place in the mass spectrometer [12, 24]. The nomenclature used in the following discussion for description of the observed fragment ions is explained in Figure 3. The most intense signals in the spectra are due to a second cleavage of a glycosidic bond (leading to isobaric C or Z fragment ions), accompanied by partial losses of the side chain (isobaric E or W ions and isobaric D or V ions). Both spectra are similar in that they show the complete series of C/Z and E/W fragment ions. The fragmentation pattern of the 6-isomer is characterized by D/V cleavages (glycosidic bond cleavage plus side chain fragmentation, indicated as $\downarrow$ in Figure 2a). A corresponding series of cleavages is observed for the 2-isomer, but is detected as sodiated rather than protonated ions (indicated as ■ in Figure 2b). Structures and observed fragmentations for the two cyclodextrin derivatives are shown in Figure 3. Fragment ions of types D/V, E/W and C/Z, as described above, do not contain information about the modification sites within the derivatized glucose ring. In addition to these fragmentations, however, ring cleavage reactions were observed in the PSD spectra, which can be used to identify structural isomers [24]. The most important difference between the two spectra thus is the occurrence of fragment ion

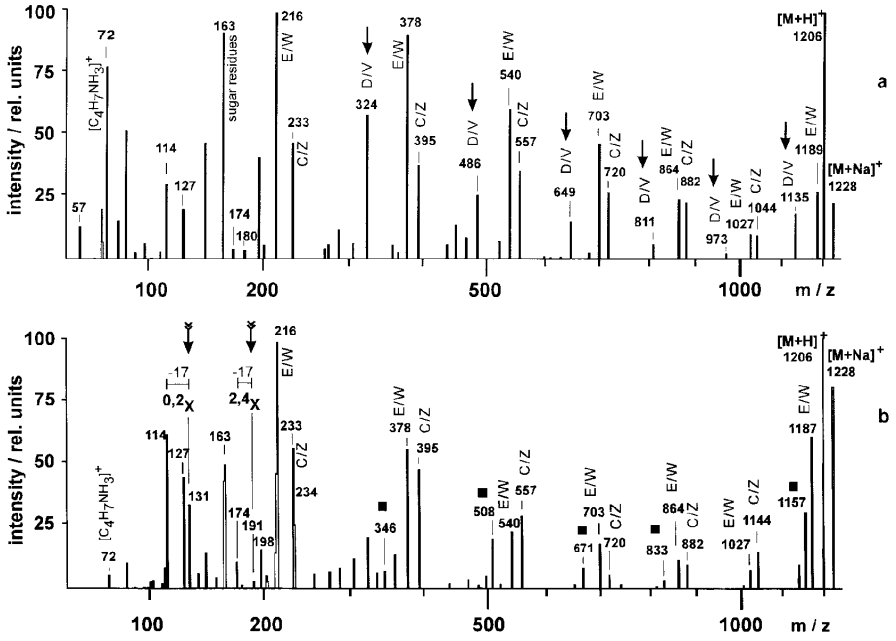


Figure 2. (a) PSD-spectra of mono-6-(ω-aminobutylamino)-2-deoxy-β-cyclodextrin, (b) mono-6-(ω-aminobutylamino)-2-deoxy-β-cyclodextrin **5b**.

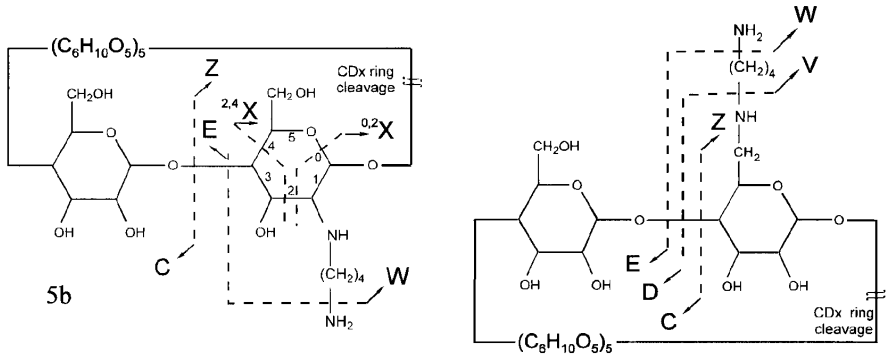


Figure 3. Structure and fragmentation scheme of mono-2-(ω-aminobutylamino)-2-deoxy-β-cyclodextrin **5b** and mono-6-(ω-aminobutylamino)-6-deoxy-β-cyclodextrin.

signals at masses 131 and 191 u in the 2-isomer (Figure 2b). These fragment ions can be interpreted as retro-aldol reaction products ^{0,2}X and ^{2,4}X as shown in Figure 4, confirming the expected derivatization in position 2 of one glucose ring. These ions occur in the spectrum of the 2-isomer due to the fact that the aminobutyl residue contains two nucleophilic nitrogen atoms which are able to localize the positive charge (i.e. the additional proton). For the 6-isomer, the corresponding fragmentation reactions do not lead to charged fragments since the fragments do

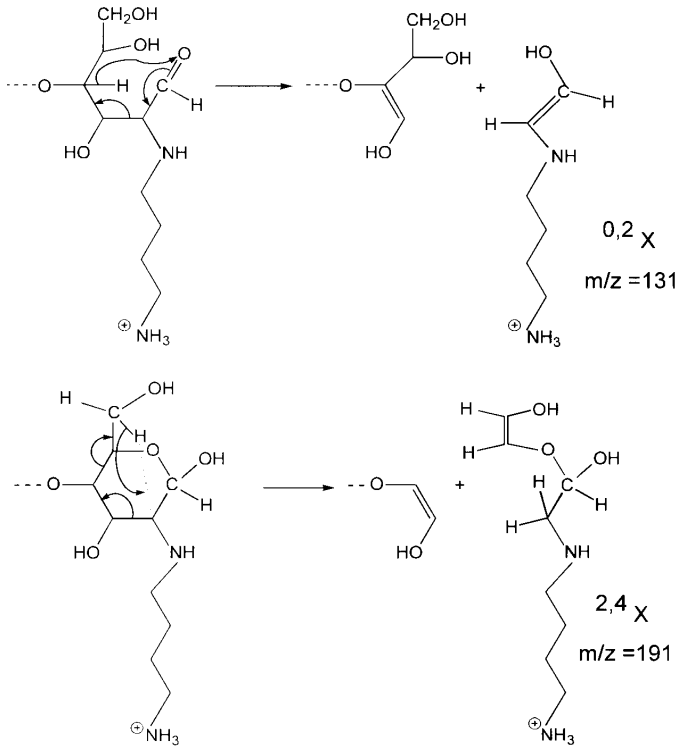


Figure 4. Structure of fragment ions with m/z 131 and 191.

not contain the charge carrying amino groups. Other ring fragmentation reactions of the 6-isomer, which would contain the amino groups instead, were not observed. This might be due to the fact, that for the 6-isomer the aminobutyl group is cleaved off more easily as indicated by higher intensities of the D/V fragment ions, and is thus not accessible for second order ring fragmentation reactions.

PSD-MALDI-MS data thus allow one to distinguish between the two isomers of the cyclodextrin monomer and indicate that **5b** is indeed the cyclodextrin derivative modified in position 2 of one glucose ring.

3.3. BINDING CONSTANTS

The binding properties of the synthesized β-cyclodextrin dimers were determined by fluorescence spectroscopy. 6-(p-Toluidino)-2-naphthalenesulfonic acid (TNS) was used as a fluorescent guest molecule. TNS shows negligible fluorescence in aqueous solutions, but displays strong fluorescence when incorporated in the hydrophobic cavity of a cyclodextrin. TNS is known to form 1 : 1 complexes with β-cyclodextrin dimers [16]. The addition of increasing amounts of β-cyclodextrin dimers to an aqueous TNS solution enhanced the fluorescence intensity remark-

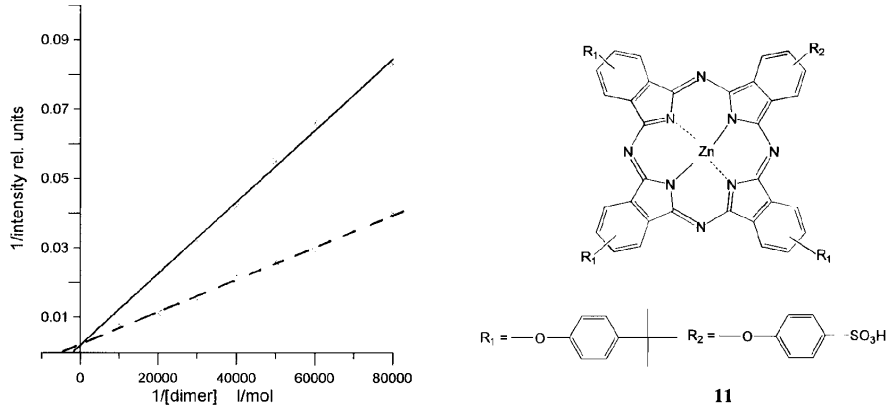


Figure 5. Double reciprocal plot for TNS fluorescence intensities in β-cyclodextrin dimer **7c** solution, [TNS] = 1.0×10^{-5} mol/L. First run (- - - -) with guest TNS, second run (—-) with competitor: Zn-tri-*t*-butylphenoxy-mono-sulfophenoxy-phthalocyanine [25] [**11**] = 1.0×10^{-7} mol/L.

Table II. Binding constants [L/mol] of cyclodextrins with porphyrinoid derivatives.

Hosts	Guests	Porphyrinoid derivatives		
Cyclodextrins	TNS	10	11	12
7a	$3.6 \pm 0.2 \times 10^3$	$4.5 \pm 1.3 \times 10^5$	$3.3 \pm 0.5 \times 10^5$	$2.1 \pm 0.5 \times 10^5$
7b	$3.8 \pm 0.3 \times 10^3$	$3.1 \pm 0.8 \times 10^5$	$1.3 \pm 0.3 \times 10^5$	$6.3 \pm 1.9 \times 10^5$
7c	$5.5 \pm 0.3 \times 10^3$	$1.3 \pm 0.5 \times 10^5$	$1.5 \pm 0.3 \times 10^7$	$8.4 \pm 1.8 \times 10^5$
7d	$5.6 \pm 0.2 \times 10^3$	$1.4 \pm 0.3 \times 10^5$	$4.1 \pm 0.9 \times 10^6$	$8.7 \pm 0.4 \times 10^5$
8a	$8.3 \pm 0.4 \times 10^3$	$5.8 \pm 1.7 \times 10^4$	$4.0 \pm 0.7 \times 10^6$	$5.0 \pm 0.5 \times 10^5$
8b	$5.8 \pm 0.2 \times 10^3$	n. d.	$1.4 \pm 0.4 \times 10^6$	$2.4 \pm 0.4 \times 10^5$
Me-β-CD	$8.3 \pm 0.2 \times 10^3$	n. d.	$6.8 \pm 1.3 \times 10^4$	n. d.

[d] Not determined.

ably. The binding constants of β-cyclodextrin dimers with porphyrinoid derivatives were determined by means of competitive spectrofluorometry. Adding a new guest molecule (the porphyrinoid derivative) to the TNS–cyclodextrin solution greatly reduced fluorescence intensity. Double reciprocal plots of titration data from the first run (with guest TNS) and from the second run (with competitor) gave straight lines (Figure 5). The linearity of these plots confirmed that the dimer and TNS formed 1 : 1 complexes.

The binding constants of the inclusion complexes of β-cyclodextrin dimers with porphyrinoid derivatives are summarized in Table II. TNS did not bind to the β-cyclodextrin dimers as tightly as to Me-β-cyclodextrin. However, binding constants increased with decrease in the spacer lengths of β-cyclodextrin dimers.

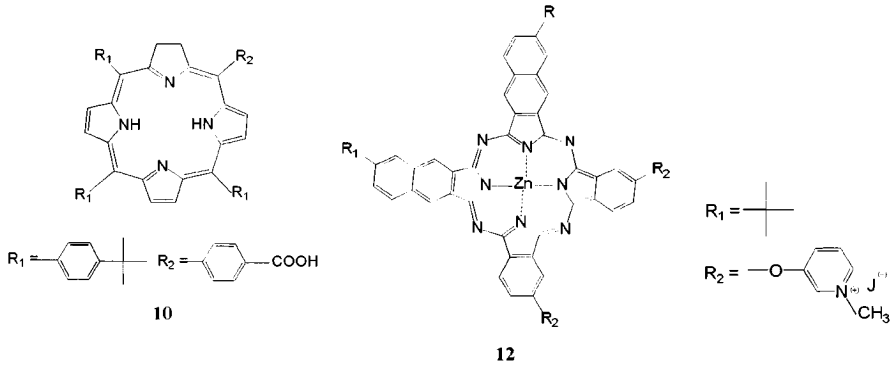


Figure 6. Porphyrinoid derivatives tri-t-butylphenyl-mono-carboxyphenyl-porphyrin **10**, bis-(3-pyridyloxy)dibenzo-di-naphtho-porphyrazinato-zinc(II)-complex **12**.

We examined the porphyrinoid derivatives **10–12** (structures shown in Figures 5 and 6) as guest molecules. They are similar in that they all are substituted with t-butylphenyl groups.

Our investigations yielded binding constants of β-cyclodextrin dimer inclusion complexes with porphyrinoid derivatives **10–12** ranging from 10^5 to 10^7 L/mol. The largest binding constant found was that of Zn-tri-t-butylphenoxy-mono-sulfophenoxy-phthalocyanine **11** in combination with dimer **7c**. Secondary face linked β-cyclodextrin dimers **7a–d** differ in their binding abilities from primary face linked β-cyclodextrin dimers **8a–b**. This was concluded from the emission maximum ($\lambda^{\mathrm{F}}_{\mathrm{max}}$) of the TNS fluorescence. The position of this emission maximum provides information about the polarity of the environment [16]. The value of $\lambda^{\mathrm{F}}_{\mathrm{max}}$ for **7a–d** ranged from 419 to 428 nm, the corresponding value for **8a–b** was 448 nm. (For comparison: the value of $\lambda^{\mathrm{F}}_{\mathrm{max}}$ for Me-β-cyclodextrin is 453 nm.) The observed blue shift for dimers **7a–d** indicates a pronounced incorporation of the TNS molecules into secondary face linked dimers.

3.4. THEORETICAL STUDIES

To date, application of theoretical approaches to host–guest complexes with cyclodextrin monomers has been rather limited [26]. The goal of our theoretical studies was to find possible correlations between calculated geometries and theoretical energy gains due to complexation of inclusion compounds on the one hand and experimentally measured values of binding constants and optimum spacer lengths on the other. Molecular mechanics calculations and molecular dynamic simulations were performed on a personal computer with HyperChem software (Hypercube Inc., Canada). The geometry optimizations (guest and host alone, then together) were made using the MM+ force field. The calculations did not take account of any water molecules (modeling in the gas phase), except for one complex with a secondary face linked cyclodextrin dimer **7** and C(9)-spacer which, for comparis-

Table III. Calculated steric and gas phase binding energies of inclusion complexes. Steric energy E_s (energy value calculated by HyperChem), binding energy E_b $\sum E_{\text{scomponents}} - E_{\text{scomplex}}$.

Host	n	m	Guest	E_s (kJ/mol)	E_b (kJ/mol)
7	2	3	**11**	1542.38	246.10
7	2	4	**11**	1546.69	237.77
7	3	3	**11**	1623.22	216.25
8	2	3	**11**	1765.28	179.53
8	2	4	**11**	1798.15	164.04
8	3	3	**11**	1776.79	184.47

on, was optimized in a periodic box with about 1000 water molecules. This way, we have found only little differences to the preceding calculation. Particular care was taken to choose highly symmetrical starting structures of the dye–cyclodextrin dimer complexes for optimization. At least three starting conformations were generated and optimized. In most cases the results were similar, therefore the lowest calculated steric energy was used to compute the gas-phase binding energies ($\sum E_{\text{components}} - E_{\text{complex}}$) [27, 28]. All presented results may be improved by taking into account the influence of water. The calculated steric and binding energies (Table III) of complexes with secondary face linked dimers **7** (a calculated structure is shown in Figure 7) were generally higher than with dimers linked at their primary face **8**. Varying spacer lengths of 9 to 11 C-atoms secondary face linked dimers showed a decreasing binding energy with increasing spacer lengths. The binding energies of dimers with C(9)–C(11) spacers on the primary face varied only scarcely. The spacers of these dimers were more strongly bent than those of dimers **7**.

C(12)-, C(13)- and C(14)-spacers in dimers **7** linked on the secondary face showed a very high flexibility. Frequently, we observed a considerable shortening by bending, coupled with twisting of the cyclodextrin molecules around the spacer. The binding energies showed corresponding or lower values as for complexes with C(9)–C(11) spacers. During 10 ps molecular dynamic simulations (1.5 ps 50–300 K; 8.5 ps T = 300 K) at least one t-butylphenoxy group of the guest **11** left the cyclodextrin cavity if the spacer had more than 11 C-atoms.

4. Discussion

Carrier systems for the application of drugs have been developed in order to achieve an inert drug for photodynamic therapy. As carrier systems, β-cyclodextrin dimers linked at their primary and secondary faces by spacers of varying lengths were synthesized and characterized by MALDI-MS. Using a multistep procedure involving reaction of ω-aminoalkylamino substituted β-cyclodextrin monomers

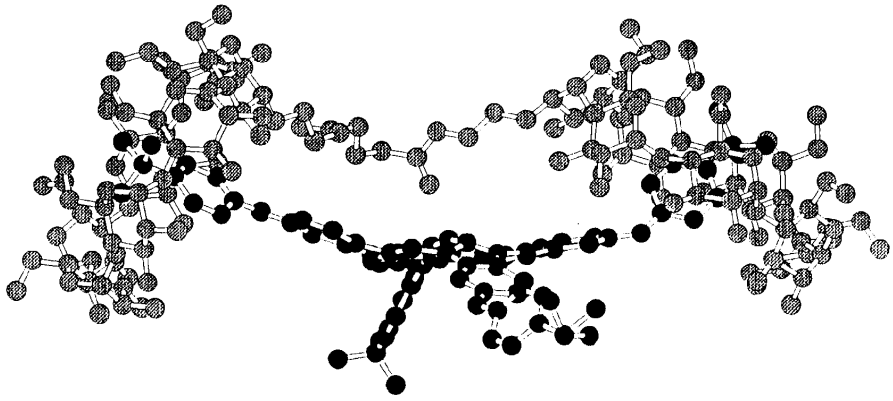


Figure 7. Calculated structure for the complex between secondary face linked cyclodextrin dimer **7** (n = 2, m = 3) and Zn-tri-t-butylphenoxy-monosulfophenoxy-phthalocyanine **11**. Hydrogens omitted.

5a,b and **6a** with N-hydroxysuccinimide di-esters **9a–c**, yielded a convenient method to synthesize various β-cyclodextrin dimers with different spacer lengths through a varying combination of these compounds. Structural proof of the newly synthesized β-cyclodextrin dimers was provided by using PSD-MALDI-MS. This was the first application of PSD-MALDI-MS in structural analysis of cyclodextrins. The characteristic differences in spectra of 2- and 6-substituted β-cyclodextrins provided important information about their structure. PSD-MALDI spectra yielded two characteristic fragment ions for the cyclodextrin derivative substituted at position 2, whereas analysis of 6-substituted cyclodextrin showed no isomer-specific fragment ions at all, probably due to a premature cleavage of the aminobutyl residue. The structure of the β-cyclodextrin dimers were not analysed by PSD-MALDI-MS, due to the evidence of the isomeric purities of the precursors (mono-ω-aminoalkylamino-β-cyclodextrins). The advantages of this method are femtomolar sensitivity, investigation of crude products as reaction control and fast analysis. In contrast, NMR spectroscopy required a long scanning time and a large amount of sample for characterizing substituted cyclodextrins.

The primary and secondary face linked dimers were investigated with respect to their binding ability to porphyrinoid photosensitizers. Breslow and coworkers [7] showed that guest molecules with t-butylphenyl groups bind tightly into the cavity of β-cyclodextrins. It was shown that all secondary face linked dimers **7a–d** exhibit larger binding constants with porphyrinoid derivatives as do primary face linked dimers **8a–b**. This was also confirmed by the calculated gas-phase binding energies.

The largest binding constant with a value of $1.5 + 0.3 \pm 10^7$ L/mol was achieved with dimer **7c** as host and Zn-tri-t-butylphenoxy-mono-sulfophenoxy-phthalocyanine **11** as guest. However, calculation of binding constants with competitive spectrofluorometry can only give apparent binding constants, due to the

indirect determination method. By monitoring the change in fluorescence intensity of porphyrinoid molecules with a red-sensitive spectrofluorometer, it would be possible to determine binding constants directly. Fluorescence spectroscopy is a very sensitive method for observing the encapsulation of porphyrinoid derivatives [29], whereas the use of UV/Vis spectroscopy often contains errors regarding aggregation phenomena of porphyrin derivatives [10].

It has been proposed that the forces that drive the formation of inclusion complexes are a combination of van der Waals attractions, electrostatic interactions, conformational changes in host or guest molecules and the interaction of the cyclodextrin and guests with solvents [26]. Several authors have presented results about MO calculations or NMR spectroscopic investigations of inclusion complexes in regard to their guest orientation [30–33]. Lawrence and coworkers [32, 33] described the penetration of phenyl-substituted porphyrins into the β-cyclodextrin molecule through the secondary face. This is most likely the reason for the larger binding constants of the secondary face linked dimers with porphyrinoid guests.

As a result of synthesizing β-cyclodextrin dimers of varying spacer lengths, we were able to establish the optimal spacer length for strong binding to each porphyrinoid derivative. Using these results, it was possible to outline a β-cyclodextrin dimer suitable for use as a carrier for photosensitizers in PDT. For the Zn-tri-t-butylphenoxy-mono-sulfophenoxy-phthalocyanine **11** as sensitizer, β-cyclodextrin dimer **7c** appears to be the best carrier, whereas, for the tri-t-butylphenyl-monocarboxyphenyl-porphyrin **10** a β-cyclodextrin dimer with shorter spacer seems to be more effective. All investigated inclusion complexes with binding constants ranging from 10^5 to 10^7 L/mol are considerably stable. The high stability of these inclusion complexes prevents photosensitizer transport on the lipoprotein pathway. Stability and inertness of the complexes vs. plasma proteins were demonstrated by the spectral differences of the inclusion complex and the lipoprotein complexed drug in absorption spectroscopy [34]. Having successfully developed inert inclusion complexes of porphyrinoid photosensitizers and β-cyclodextrin dimers, the next step will be the tumor targeting with functionalized photosensitizers and antibodies.

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