JOURNAL OF PHYSICAL ORGANIC CHEMISTRY
J. Phys. Org. Chem. 2001; **14:** 159–170

The complexation of peptides by aminocyclodextrins

Frank Hacket, Svetlana Simova[†] and Hans-Jörg Schneider*

FR Organische Chemie der Universität des Saarlandes, D-66041 Saarbrücken, Germany

Received 24 August 2000; revised 5 November 2000; accepted 15 November 2000

epoc ABSTRACT: Four β-cyclodextrins (CDs) were prepared bearing either an (*N*,*N*-dimethylamino)propylamino group (**1**), an (*N*-methyl)-piperazino group (**2**) or a benzylamino group (**3**), or seven methylamino susbtituents (**4**). Association constants *K* in water with di- and tripeptides reach up to 200 M^{-1}, and after protection at the *N*-terminus up to 680 M^{-1}. Appreciable binding occurs only in the presence of lipophilic amino acid side-chains, with preference for this at the *C*-terminus. A moderate sequence and side-chain selectivity is observed with **1**, **2** and **3**, but less so with the highly charged **4** where ion pairing dominates. Detailed NMR analyses with advanced techniques including T-ROESY and GHSQC allow full assigment of most ^{1}H and ^{13}C signals, with extraction of many substituent and complexation induced shifts changes (SIS and CIS values, respectively). The CIS values and NOE cross peaks from ROESY experiments provide for insight into the binding modes of selected complexes, indicating, e.g., the simultaneous presence of complexes with a peptide phenyl unit approaching from both the narrow and the wide side of the CD cavity. With **3** one observes self-inclusion of the pendant phenyl ring within the cavity, and its replacement by analytes such as peptides, or by adamantanecarboxylic acid. The inclusion modes are illustrated with force field simulated structures and many NMR spectra, which are made available in electronic supplements. Copyright © 2001 John Wiley & Sons, Ltd.

Additional material for this paper is available from the epoc website at http://www.wiley.com/epoc: (NMR shift tables; 3D coordinates for molecular structures; 1D- and 2D-NMR spectra)

KEYWORDS: peptides; complexation; aminocyclodextrins; NMR analyses; force field calculations

INTRODUCTION

The binding of lipophilic substrates inside the cavity of cyclodextrins (CDs) has been the focus of many investigations.[1] Surprisingly, the complexation of peptides has until now received little attention, in contrast to many other studies with bioactive guest compounds, including amino acids.[2] Non-covalent interactions with peptides are of paramount importance for understanding mechanisms of protein folding, but also for, e.g., pharmaceutical applications. The low toxicity of CDs and their increasingly economic availability makes these host compounds particularly amenable for, e.g., drug protection and delivery. Also for these reasons the design of suitable artificial peptide receptors, which are less developed in comparison with host compounds for nucleotides, should be pursued on the basis of CDs. The present study addressed this need, with an emphasis on a better understanding of the underlying complex structures in the functional state of solution.

Most of the literature on CD complexes is restricted to measurements of association constants, including many thermodynamic studies. The wealth of information from these data, however, has not led to a consistent picture of the relevant binding mechanisms with CDs, which are more diverse than with other host compounds, and still a matter of significant controvery.[2,3] Often it is not even known whether and which lipophilic parts of the substrate are bound within the CD cavity. It has been shown that it not necessarily the geometric fit that determines this, against chemical intuition or predictions from computer-aided molecular modelling.[4] The obvious remedy here is the use of NMR methods, which in view of the underlying complex CD spin systems require application of high fields and of advanced NMR techniques.[5] The complications are particularly large if the inherent symmetry of the CD moiety is distorted by substitution; such complexes are the main focus of the present study.

Complexes of native, unsubstituted CDs with peptides are very weak, which might be one reason why they have rarely been studied. We decided to use aminocyclodextrins, which have been found to be very effective host compounds for nucleotides and even nucleosides.[6] The presence of a positively charged side-group in the CD can

**Correspondence to:* H.-J. Schneider, FR Organische Chemie der Universität des Saarlandes, D-66041 Saarbrücken, Germany.
E-mail: ch12hs@rz.uni-sb.de
[†]On leave from the Institute of Organic Chemistry of the Bulgarian Academy of Sciences.
Contract/grant sponsor: Deutsche Forschungegemeinschaft.
Contract/grant sponsor: Fonds der Chemischen Industrie.

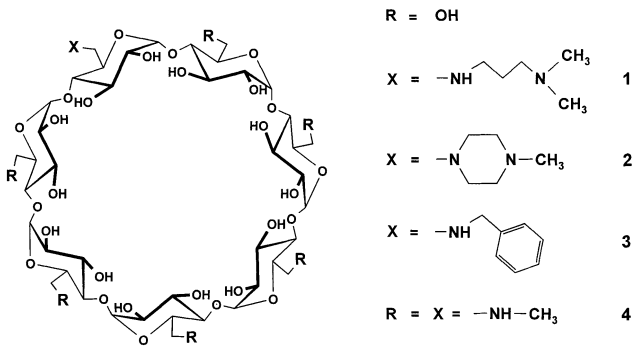

Figure 1. Investigated amino-CDs

lead to relatively strong salt bridges with the *C*-terminus of peptides; together with the lipophilic side-chain part of amino acids enhanced and eventually sequence-selective recognition might be feasible. CDs with pendant aromatic side-chains have been used in particular by Ueno and co-workers for fluorimetric assays of many analytes.[7] Detailed NMR analyses[7] with such host compounds have already provided clear evidence for self-inclusion of the pendant aromatic side-chain within the CD cavity, which can be expelled by addition of stonger binding analytes.

RESULTS AND DISCUSSION

The amino-CDs investigated are shown in Fig. 1.

Syntheses, protonation state

Monoaminocyclodextrins **1**–**3** were prepared from the corresponding mono-6-*O*-tosylate by nucleophilic substitution (see Experimental section). The monotosylate was obtained under aqueous conditions using toluenesulfonyl chloride (T. Ikeda, Tokyo Institute of Technology, personal communication) giving the same yields and purity reported before for pyridine as solvent.[12] The recently reported[13] higher yields with *p*-toluenesulfonic anhydride as reactant were not reproducible in our hands.

The heptaamino-CD **4** was prepared according to a procedure by Breslow *et al.*[14] Instead of the β-CD-6-heptatosylate, the β-CD-6-heptaiodide was used for the nucleophilic substitution. Following the synthesis of Ashton *et al.*,[15] the iodide was prepared and converted into the desired amine **4** in a more reproducible way than that with the use of the tosylate.

The charges for the monosubstituted CD derivatives **1**–**3** were estimated from known pK_A values of the corresponding amines (see footnotes to Table 1). NMR experiments at pD = 2.4, 7.4 and 12.4, however, showed significant differences due to ligation of the amines to the CD moiety, with protonation degrees as given in Table 1

Table 1. Protonation degree and overall charges of monoamino-CDs **1**–**3** (calculated from ^{1}HNMR measurements at pD = 2.4, 7.4 and 12.4)[a–c]

CD	Protonation degree (%)	Overall charge at pD 7.4
1	N-14, 50; N-3, 90	+1.4
2	N-1, <10, N-4, 60	+0.7
3	60	+0.6

[a] At 298 K in D_2O, c(NaCl) = 1×10^{-2} mol l^{-1}, pD = 7.4 corresponds to pH = 7.0.
[b] Literature values/overall charges at pH 7.0 of the underlying unsubstituted amines from potentiometric measurements in water at 298 K (all values within 0.16 units)[36]. For **1** (1,3-diaminopropane),[35] 10.9, 9.0 /+2; for **2** (dimethylpiperazine),[35] 8.54, 4.63 /+1; for **3** (*N*-methylbenzylamine), 9.57, lowest literature value.[36]
[c] At values of pD = 2.4 and 12.4 only the fully protonated, or the fully deprotonated forms, respectively, exist. The protonation degree is determined from the ratio the differences in the chemical shifts of protons located close to the protonation site between pD = 7.4 and 12.4 to the chemical shift difference of the whole range pD = 2.4 and 12.4 (see supplementary table S_Table I).

(see also S_Table I in the supplementary material at the epoc website at http://www.wiley.com/epoc). The hepta-substituted **4** was estimated to bear 4–5 positive charges on the basis of potentiometric investigations by Hamelin *et al.*[16] with a comparable 6-heptaamino-β-CD.

Association constants

K values were measured using a competition method with the fluorescence dye 1-anilinonaphthalene-8-sulfonate (ANS), which provides a sensitive change of fluorescence emission upon addition of the substrates, also giving evidence for an intracavity inclusion mode. Evaluation of binding isotherms with known equations[17] (see Experi-

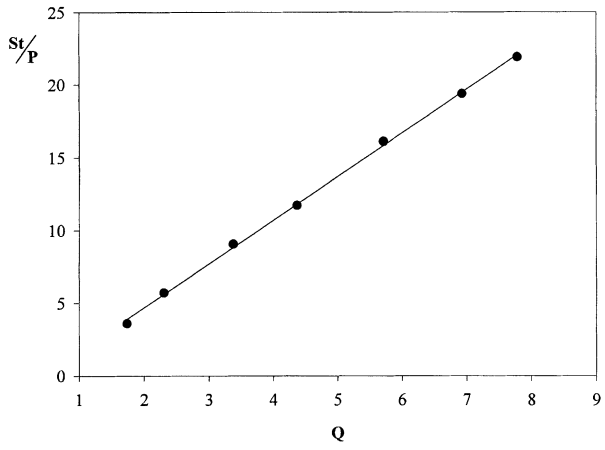

Figure 2. Fluorescence competition of the complex Ac–Gly–Phe, ANS and the cyclodextrin **4** in water, pH 7.0, $c_{(NaCl)} = 1 \times 10^{-2}$ mol l^{-1}, T = 298 K, λ_{ex} = 360 nm, λ_{em} = 500 nm (for fitting equations and parameters, see Experimental)

Table 2. Association constants, ($K_{ass.}$ (M^{-1}) and free complexation enthalpies, ΔG (kJ/mol^{-1}) of complexes with the aminocyclodextrins **1**–**4** from fluorescence titrations with 1,8-ANS in water, [pH 7.0, c(NaCl) = 1×10^{-2} mol l^{-1}, T = 298 K[a,b]

	1		**2**		**3**		**4**	
Complex	$K_{ass.}$	ΔG	$K_{ass.}$	ΔG	$K_{ass.}$	ΔG	$K_{ass.}$	ΔG
Gly–Gly–Gly[c]	<5	>−4.0	<10	>−5.6	<5	>−4	—	—
Phe–Gly–Gly	13	−6.3	19	−7.3	20	−7.4	56[e]	−10.0
Gly–Phe–Gly	23	−7.8	37	−8.9	36	−8.9	20	−7.4
Gly–Gly–Phe	50	−9.7	48	−9.6	27	−8.2	72	−10.6
Phe–Gly	22	−7.7	—	—	25	−8.0	206	−13.2
Gly–Phe	49	−9.7	—	—	35	−8.8	78[e]	−10.8
Ac–Gly–Phe	80	−10.9	—	—	53	−9.8	680	−16.2
Phe–Asp	—	—	—	—	19	−7.3	—	—
Asp–Phe	—	—	—	—	73	−10.6	—	—
Trp–Gly–Gly	10	−5.6	<20[c]	>−7.3	37	−9.3	52	−9.8
Gly–Trp–Gly[d]	—	—	—	—	—		—	—
Gly–Gly–Trp[c]	<50	>−9.7	<100	>−11.4	<50	>−9.7	<100	>−11.4
Leu–Gly–Gly	<3	>−2.7	<10[c]	>−5.6	5	−4.0	45	−9.5
Gly–Leu–Gly	18	−7.1	<10[c]	>−5.6	12	−6.2	13	−6.4
Gly–Gly–Leu	19	−7.4	21	−7.4	21	−7.5	31	−8.5

[a] Error in K <15%.
[b] $K_{ass.}$ (β-CD/Gly–Gly–Phe) <10 M^{-1}, in line with literature[37] data from UV–VIS measurements for the α-CD/Gly–Gly–Phe system ($K_{ass.}$ = 8.1–10-4 M^{-1} in water, pH 9.0).
[c] No fluorescence change observed upon adding peptide to the system 1,8-ANS/CD.
[d] Too low solubility of the peptide (<5×10^{-4}mol l^{-1}).
[e] Error <20%.

mental) yielded satisfactory linear fitting for a 1:1 stochiometry (Fig. 2) and binding constants for the amino-CDs **1**–**4** (Table 2). Limiting factors for the determinations and the errors in K were often low solubilities of the peptides; in several cases only approximate values or upper or lower limits could be given.

Affinity variations

Appreciable association of the peptides with the CDs (Table 2) was only observed in the presence of lipophilic amino acid side-chains. Thus, the tripeptide Gly–Gly–Gly showed no affinity above the detection limit with any CD host. Removal of the positive charge in a substrate by converting it into the *N*-acetyl derivative leads, as expected on the basis of ion pairing. to an increase in the association constants. This is especially so with the heptasubstituted **4**, in which the many charges lead to ion paring as the dominating factor. Here, the K value is increased by a factor of almost 10 by neutralizing the positive charge at the peptide, whereas the increase with host **1** or **3** is only moderate.

With a series of tripeptides containing always two Gly and either Phe, Trp or Leu units, we investigated the possible sequence selectivity of the host compounds. With all CD hosts except the benzyl derivative **3** the K values, as far they could be measured, are larger if the lipophilic amino acid is at the *C*-terminus—this location allows simultaneous contact of the carboxylate to the CD ammonium groups and the aromatic side-chain with the CD cavity. NMR data with another peptide (see below) support this inclusion mode. The alternative dipeptides Phe–Gly and Gly–Phe show, in line with the discussed inclusion mode, within error the same K values as the tripeptide as long as the terminal sequence is Gly–Phe, but a much larger constant with the heptamino-CD and Phe–Gly, with a different sequence. In the structure with this dipeptide, optimum ion pairing is not hindered by the interactions between the lipophilic parts of the host and guest. The presence of an additional anionic charge in the Asp-containing peptides leads to a moderate affinity increase in comparison with Phe–Gly or Gly–Phe, and this only with the Asp–Phe-sequence.

Selectivity with respect to the nature of the amino acid side-chain is again only moderate, but in line with expectations based on the increasing lipophilicity. Again, the dominating ion pairing with the highly charged host **4** leads to decreased selectivity.

Host **3** differs from the others by the possibility of competing self-inclusion of the pendant phenyl unit, in line with related literature systems of Ueno and co-workers.[7] This geometry is established by the observed NMR shifts and NOEs, and also by an experiment with added adamantanecarboxylic acid and with the added peptides, which expel the phenyl unit from the cavity (see below). NMR analyses also exclude the presence of intermolecular associations at the measuring conditions (as indicated in Fig. 3). The intramolecular occupation of the CD cavity by the phenyl ring in **3** leads to an affinity decrease towards peptides, which in view of the K values

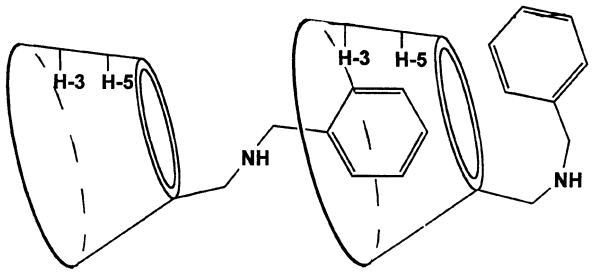

Figure 3. Complex geometry for intermolecular inclusion of the benzyl ring in **3**

obviously is only partially compensated by interactions between the expelled phenyl ring outside the CD cavity and lipophilic parts of the peptides.

NMR analyses

Assignment and measuring methods. Owing to the complexation the chemical shifts of *both* cyclodextrin and peptide protons change, so that especially in the case of the mono-substituted derivatives separate assignment for the complex has to be made, taking into account the stoichiometry and the binding energetics. Characteristic patterns for α-glucosidic protons are a doublet with a constant around 4 Hz for H-1, a double doublet (10 and 4 Hz) for H-2 and triplets with a coupling constant around 10 Hz for H-3 and H-4, the former usually resonating at a 0.3–0.4 ppm lower field. Only the pattern of the spin system comprising H-5 and H-6a,b protons usually changes owing to substitution and/or inclusion shielding effects. Unresolved or second-order spectra for these protons were observed even at 500 MHz, depending on the chemical shift difference between them. These signals often overlap with some others; determination of the coupling constants ($^3J_{5,6}$) is difficult, thus precluding determination of the preferred conformation of the substituents with respect to the glucose ring. Well separated signals are only observed with substituents with large anisotropy or field effects. For the assignment of these protons measurement of a 2D C–H correlation such as HSQC or GHSQC is more advisable than the usually first acquired COSY. Determination of the $^3J_{5,6}$ coupling constants could be achieved in strongly coupled systems with the aid of the coupled version of the HSQC experiment.[18] In the hetero-correlation experiment the carbon NMR spectrum is obtained in addition; with this, the assignment of the closely resonating C-2, C-3 and C-5 atoms could be made unambiguously.

A particular problem with the heptasubstituted compounds is their low solubility in water. Protonation of the nitrogen atoms leads to better solubility. However, complicated spectra are obtained at the pH values between 5 and 7 usually necessary for complexation studies, owing to the different protonation degree and the pH dependence especially of the H-5 and H-6 signals. DMSO is usually a better solvent in this case for identification purposes.

Observation of different shifts in monosubstituted CDs does not necessarily indicate restricted motions, but is in line also with the existence of predominant conformations.[16,19] For all compounds and complexes acquisition of 2D TOCSY, GHSQC and T-ROESY was necessary. The area between 4.1 and 3.5 ppm is always a superposition of many signals and only occasionally contains a signal with the necessary shielding separation. For the investigated compounds this was the case, when either a phenyl ring is fully or partially immersed in the CyD cavity, owing to the anisotropy effects of the corresponding ring current and/or when the 6-amino nitrogen is fully protonated.

The 2D TOCSY spectra 4 afford separate sets of signals for the different glucose units. From the traces of the corresponding anomeric protons, assignment of the remaining signals can be made, taking into account the

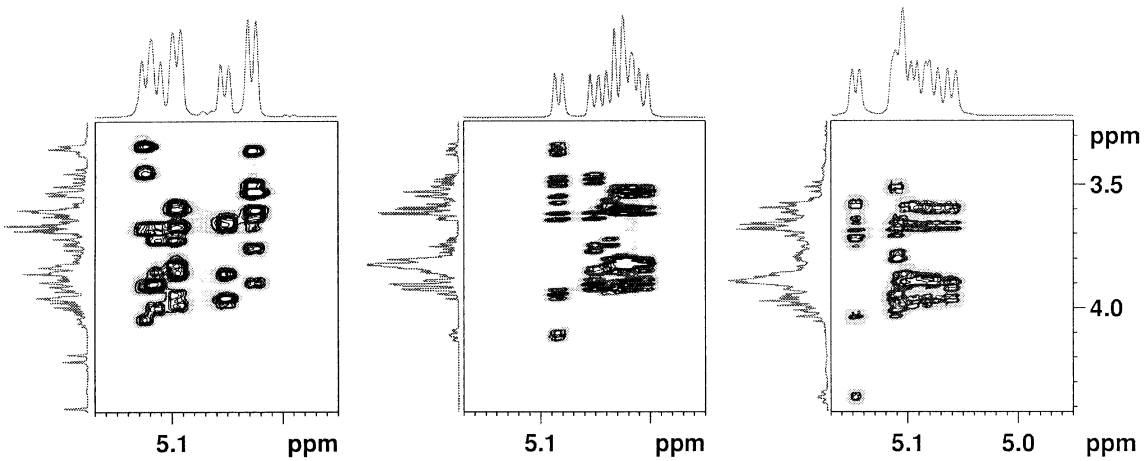

Figure 4. TOCSY NMR spectra of the **1**–**3** (only CyD protons are shown), illustrating the proton shift dispersion due to different substitution

coupling pattern of the signals. Owing to the different resolution in both directions, traces and columns usually give supplementary information for the overlapping regions. When severe overlapping of more signals occurs and/or when second-order effects due to small chemical shift differences between vicinal protons (mostly H-4, H-5 and/or H-5, H-6) arise, 1D TOCSY experiments with different mixing times facilitate unambiguous assignments. For the compounds studied, COSY-type spectra even in the DQF (double quantum filter) version of the experiment gave severe overlapping, and were therefore of little use.

In the second assignment step through-space connectivities between the different glucose units are established. We found it most useful to measure T-ROESY spectra with an 800 ms mixing time, as with this version the artifacts (*J*-cross talk, TOCSY transfer, etc.) are minimized and the intensities of the cross peaks are maximized. The main cross peaks are always observed between the anomeric H-1 protons and H-2 protons, belonging to the same glucose ring, and H-4 protons, belonging to adjacent glucose rings. The intensities of both types of cross peaks are comparable, corresponding to an average distance of 2.4 Å, according to the minimized structures (CHARMm/QUANTA V. 4.0[20]). Large cross peaks are always observed between the geminal non-equivalent H-6 protons (average distance 1.8 Å) and between the 1,3- diaxial H-3/H-5 and H-2/ H-4 protons of each glucose ring (average distance 2.7 Å). Near diagonal signals are often obscured, but the absence of a cross peak of the above-mentioned type between peaks with a chemical shift difference of more than 0.1 ppm helps to avoid erroneous assignments. In the ROESY spectra we often observed also *J*-cross talk mainly between H-2 and H-3 and between H-3 and H-4; these cross peaks were used, however, also as aid for assignment.

Assignment of the ^{13}C NMR spectra is done with the help of inverse-detected heteronuclear correlation (HSQC or GHSQC).[21] Replacement of the oxygen by a nitrogen atom (or by another substituent) in the glucose ring A moves the C-6 and C-5 carbon chemical shifts to considerably higher fields than the remaining glucose carbon atoms. The correlation then allows an unambiguous assignment of the protons in the glucose ring A and thus of the whole molecule, taking into account the through-bond connectivity from the TOCSY and the through-space connectivity from the ROESY spectra. The heterocorrelation experiments are also useful for determination and/or confirmation of some difficult proton assignments. Besides the nuclei in the substituted ring A, which are usually easy to assign, the remaining carbon atoms resonate in well-defined chemical shift ranges: 101–103 ppm for C-1, 80–85 ppm for C-4 and around 61 ppm for C-6; only the range between 71 and 75 ppm comprises C-2, C-3 and C-5 carbon atoms. In order to achieve the necessary high resolution, we found it easy and useful for these systems to use folding[22] in the carbon dimension. For this purpose, the carbon excitation frequency was centered around 73 ppm, in the middle of the most crowded region and the sampling frequency for the carbons was adjusted to a spectral width of 10 ppm, with a very high resolution (Fig. 5). Under these conditions the C-1/H-1 pairs are folded three times. However, owing to the separate region of the anomeric proton shifts, which are very well resolved, they could be phased separately. The signals for C-6/H-6 and C-4/H-4 are folded once, with some overlap. In this case, recalculation of the region and acquisition of a new spectrum are inevitable. Linear prediction in F_1 was always used for achieving of a better resolution in the carbon dimension with a significant signal-to-noise ratio increase.[23]

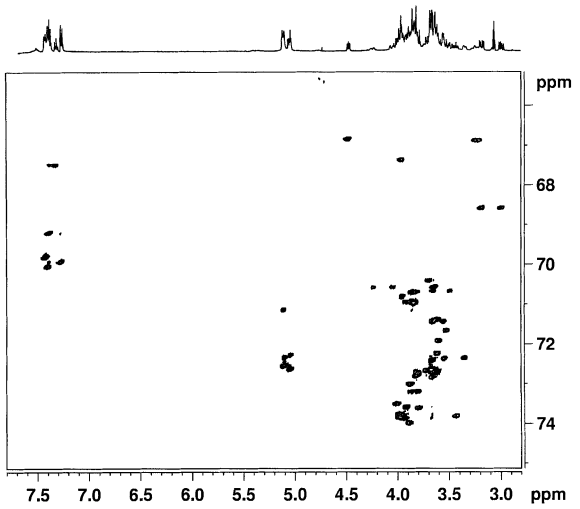

Figure 5. Inverse heteronuclear C–H correlation (GHSQC) NMR spectrum of the complex of **3** with Ac–Phe–Gly, acquired with high resolution in the second dimension (SW1 = 10 ppm)

Substituent-induced 1H NMR shifts (H-SIS). In the supplementary Tables S_Table II and III the calculated substituent-induced proton and carbon NMR shifts of compounds **1**–**4** are given; Figure 4 shows the CD protons parts of the TOCSY spectra of the compounds **1**–**3**. The dispersion of the anomeric protons is 0.09–0.14 -ppm, although these protons are at a considerable distance from the substitution site. The non-anomeric protons show two different patterns depending on the substitution. For the fully protonated compounds **1** and **2** (at pD = 2.4), having an aliphatic pendant group, distinct signals only for the protons of rings A, B, G and F were identified, whereas the protons of rings C, D and E possess nearly identical chemical shifts. It should be noted that when the nitrogen atom in ring A is not protonated, no appreciable spread of the chemical shifts for the individual glucose protons is observed. A hydrogen bond between protons of the protonated nitrogen at ring A and nearby oxygen atoms may be responsible here.[24] Our shift data and the minimized

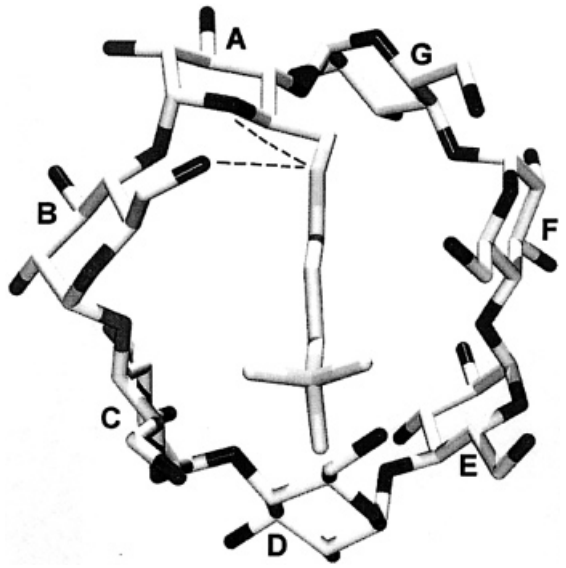

Figure 6. Minimized structure of **1**. The proximity of the protonated nitrogen atom to O 6 of ring B and O in ring A can be seen. Protons are omitted for a better view

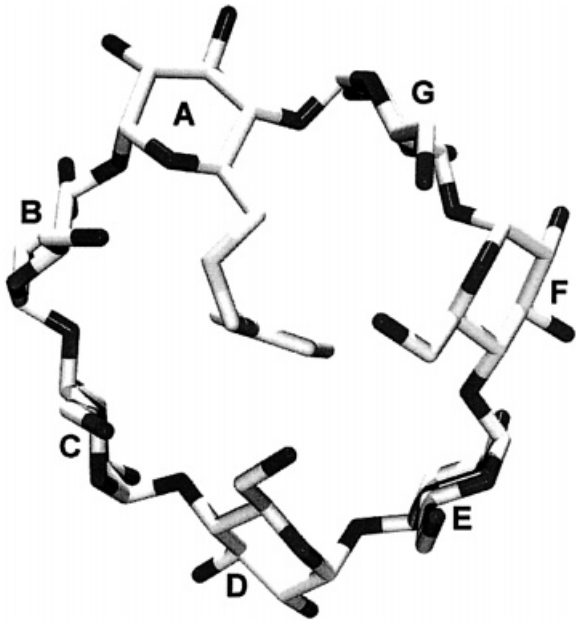

Figure 8. Preferred conformation for the benzyl ring of **3** in the CyD cavity according to the NMR data

structure (Hyperchem 5.02) show that the protonated nitrogen is located near ring B, and hydrogen bonds could exist between it and both the O atom in ring A and also the O-6 of ring B (see Fig. 6). At pH 7.00, where this nitrogen atom is protonated to a lesser extent, only a small dispersion of shifts in the unsubstituted rings is observed, precluding their unambiguous assignment.

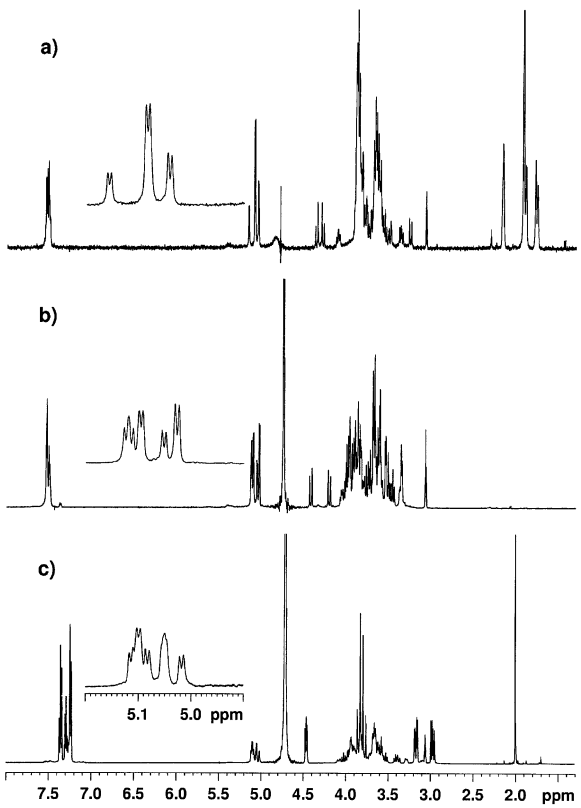

Figure 7. ^{1}H NMR Spectra of **3** (b) and its complexes with (a) ADCA and (c) with Ac–Gly–Phe

Self-inclusion of the benzyl ring in the CD cavity of 3

Dilution experiments at constant pD = 7.4 in the concentration range 1×10^{-2}–5×10^{-4} M do not show any chemical shift variation, indicating no intermolecular cavity inclusion of the phenyl ring (Fig. 3). Figure 9 illustrates the characteristic dispersion of the chemical shifts of all protons, especially of the H-5 and H-6 protons, which indicate inclusion of the phenyl ring from the primary side. This is supported by the observed NOEs between the phenyl protons and some H-5 and H-3 protons (rings A, B, E and F), disappearing on addition of

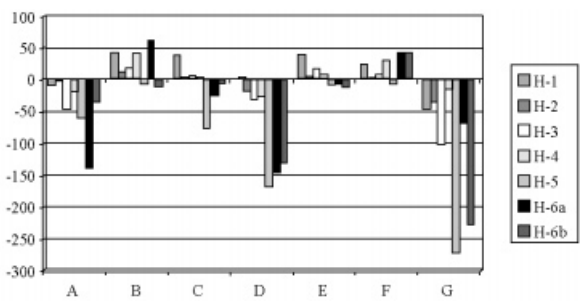

Figure 9. Shielding differences between **3** and **1** showing the net chemical shift effect arising from the introduction of the benzyl ring in the CD cavity

Table 3. Shielding differences between **3** and **1** for the glucose units A–G ($\Delta\delta \times 10^{-2}$ in ppm) at $T = 300$ K in D_2O[a,b]

Proton	A	B	C	D	E	F	G
H-1	−1.5	8.4	7.3	0.2	8.0	5.1	−8.7
H-2	−0.3	2.4	1.0	−2.4	1.0	1.0	−6.6
H-3	−8.9	3.6	1.3	3.6	−5.7	2.0	−19.6
H-4	−3.4	8.4	0.8	−5.0	1.7	6.2	−2.8
H-5	−11.6	−1.1	−15.0	−33.0	−1.0	−1.0	−53.7
H-6a	−27.3	12.5	−5.0	−29.0	−1.0	9.0	−13.1
H-6b	−6.7	−2.0	−1.0	−26.0	−2.0	9.0	−45.0

[a] c(**1**, **3**) = 2.0×10^{-2} mol l^{-1}, c(NaCl) = 1.0×10^{-2} mol l^{-1}, pD = 2.4.
[b] $\Delta\delta$ (Hz) = [δ(**3**) − (δ(**1**)]; negative values correspond to shielding and positive to deshielding.

an equimolar quantity of adamantane-1-carboxylic acid (ADCA) as competing guest (see also Fig. 7). More detailed analyses of the chemical shift data reveal that the benzyl ring has a preferred conformation in the CD cavity, since the largest shieldings are observed for the protons of the oppositely lying rings G and D (see Figs 7 and 8).

In this conformation, the protonated nitrogen atom points in the direction of ring B and can be involved in hydrogen bonding analogous to that in **1** and **2**. However, only the chemical shifts of the protons in ring A show a pronounced pH dependence (see supplementary table S_Table I), indicating that the preferred conformation of the phenyl ring does not change with pH. From the differences in the chemical shifts of **3** and **1** the net chemical shift effect arising from the ring current effect could be calculated, as shown in Table 3 and Fig. 9. Literature data on compounds with an aromatic pendant group at the primary CD side[7,25–27] (see also supplementary table S_Table IV) show that the largest chemical shift dispersion of identical atoms in the individual glucose rings is observed for the H-6 atoms, located near the substituent, and also for the inner-cavity H-5 protons. A characteristic dispersion is also observed for the H-3 protons, whereas for the other more distant protons H-1, H-2 and H-4, smaller but measurable values are also detected. It should be noted that the values for the differently substituted naphthyl (dansyl) groups[25–27] show comparable values that are about 3–4 times larger than those for the corresponding phenyl groups. The smaller values in **3** correspond to the smaller shielding cone of the phenyl group as compared with that of the naphthlene ring, and to the fact that the phenyl ring is in equilibrium between the two preferred conformations, one inside and the other outside the cavity.

^{13}C SIS values

Small but measurable dispersion of the carbon chemical shifts in the substituted compounds is detected, allowing assignment of the individual signals through heteronuclear correlation. This was possible for all carbon signals in **3**, but for **1** and **2** individual signals could be assigned only for the nuclei in rings A, B and G, which

Table 4. ^{1}H CIS values ($\Delta\delta \times 10^{-2}$ in ppm) in the complex of **1** and Ac–Gly–Phe for the glucose units A–G in D_2O at pD = 7.4 and 300 K[a]

Proton	A	B	C	D	E	F	G
H-1	−1.0	9.0 for B, C, D and E				−2.0	−4.0
H-2	<5.0	<4.0 for B, C, D, E, F and G					
H-3	−1.0	−4.0*	−9.0*	−10.0*	−15.0*	−1.0	−2.0
H-4	−3.0	<5.0 for B, C, D and E				<−4.0	<−9.0
H-5	−14.0	<−11.0* for B C, D and E				−14.0	−10.0
H-6a	19.0	<12.0*	<12.0*	<6.0*	<8.0	<10.0	<12.0*
H-6b	9.0	<−12.0	<12.0*	≈15.0–25.0	<8.0	<10.0	<6.0*
CH_2(Gly)	CH_2(Phe)	C-a	C-b	C-c	H-*o*	H-*m*	H-*p*
−6.2	−12.5	56.2	12.5	−12.5	−6.2	−12.5	−12.5
$N(CH_3)_3$	CH_3						
0.6	−6.2						

[a] Determined from the shift difference between the complex [c(**1**) = 1.2×10^{-2} mol l^{-1}, 80% in complexed state, c(Ac–Gly–Phe) = 6×10^{-2} mol l^{-1}, c(NaCl) = 1.0×10^{-2} mol l^{-1}] and **1** at pD 7.4.

Table 5. ^{1}H CIS values ($\Delta\delta \times 10^{-2}$ in ppm) in the complex of **3** and Ac–Gly–Phe for the glucose units A–G in D_2O at pD 7.4 and 300 K[a]

Proton	A	B	C	D	E	F	G
H-1	0.1	−1.1	−2.5	3.4	0.1	−2.7	−4.4
H-2	−0.8	−2.1	−4.7	3.4	−0.8	−3.7	−1.6
H-3	6.3	2.6	−6.3	−1.8	−4.0	−3.3	8.1
H-4	1.5	−2.9	−2.3	3.8	6.6	0	−1.6
H-5	−2.1	<−5.5	−7.4	8.2	<3.2	−20.1	−17.0
H-6a	1.1	<−5.5	−4.1	5.5	<8.0	2.7	−17.9
H-6b	−1.4	<−5.5	−4.1	5.5	<2.3	2.7	−24.2

[a] Determined from the shift difference between the complex [c(**3**) = 1.2×10^{-2} mol l^{-1}, 73% in the complexed state, c(Ac–Gly–Phe) = 6×10^{-2}mol l^{-1}, c(NaCl) = 1.0×10^{-2} mol l^{-1} and **3** at pD 7.4.

give separate signals especially for C-1, C-4 and C-6. It is difficult to explain these small differences because the effects due to conformational distortions, which are visible particularly in ^{13}C shift variations, are of the same order of magnitude as field and anisotropy effects. It should be noted that in all compounds the carbon atoms C-3 resonate always at slightly lower fields than the usually overlapped C-2 and C-5 carbons, which could be used for differentiation of the corresponding protons in the complexes (see below).

NMR shift analyses of the complexes/inclusion modes

Assignment of the signals in the complexes was done in the same manner as with host compounds. The complexation induced shifts (CIS), measured for the complexes of **1**, **3** and **4** with the acetylated GlyPhe, are given in Tables 4, 5 and 6. Inclusion of the phenyl ring of Ac–Gly–Phe in **4** is obvious from the complexation induced proton shifts: the higher shielding observed for H-5 than for H-3 protons indicates that the aromatic ring is closer to the primary side, fully in line with the NOE results (see below). The ^{13}C CIS values were also determined (Table 6), but seem to give insufficient selectivity for structural elucidation.

In the peptide complexes of **1** and **3** we were not able to use the CIS values for determination of the complex mode. The CIS values could be determined only if the chemical shifts of the free and of the complexed form are known. This was not the case for **1**; the shifts of the free form could not be reliably determined because of the too heavily overlapped proton spectrum at pD = 7,4, which is the value in the complexation experiments. Large shieldings are observed for protons H-3 and H-5, located inside the cavity, and also for some of the H-6 protons, but, the values obtained for H-6 are not reliable for the above reason. The data indicate inclusion of the phenyl ring in the CyD cavity, but no conclusion on the inclusion mode could be drawn solely from shift arguments.

The CIS values for the complex of Ac–Gly–Phe with **3** (see Table 5) could be exactly determined; the observed shielding and deshielding, however, do not allow unambiguous conclusions to be drawn on the complexation mode.

Table 6. ^{1}H and ^{13}C CIS values ($\Delta\delta \times 10^{-2}$ in ppm) in the complex of **4** and Ac–Gly–Phe for the glucose units A–G in D_2O at pD 7.4 and 300 K[a]

	^{13}C CIS	^{1}H CIS
H(C)-1	−12.0	−1.0
H(C)-2	−20.0	−1.0
H(C)-3	−20.0	−7.0
H(C)-4	−7.0	−1.0
H(C)-5	−15.0	−15.0
H(C)-6a	−28.0	−1.0
H-6b		−1.0
CH_3	−40.0	1.0

[a] Determined from the shift difference between the complex [c(**4**) = 5×10^{-3} mol l^{-1}, 80% in the complexed state, c(Ac–Gly–Phe) = 1×10^{-3} mol l^{-1}, c(NaCl) = 1.0×10^{-2} mol l^{-1} and **4** at pD 7.4.

NOE analyses of the complexes

If cavity inclusion of the peptide takes place, the ROESY spectra of the complexes show intermolecular cross peaks between the *ortho*- and/or *meta*-protons of the phenyl ring and the H-3 and/or H-5 protons of the cyclodextrin host. Owing to severe overlapping, it is often not possible to separate all intracavity protons; the ranges of the H-5 and the H-3 protons are then deduced from the high-resolution HSQC experiments. The NOE data for the complexes studied are given in Table 7. A preferred inclusion of the phenyl ring from the primary side could be detected only for the complex of Ac–Gly–Phe with **4** (see Fig. 10). The *ortho*-protons in the phenyl ring are in the vicinity of both H-3 and H-5 CyD protons, supported also by the higher shielding of the H-5 proton (see above), whereas the *meta*-protons are close only to

Table 7. Intermolecular cross peaks in the T-ROESY spectra of the complexes with **1**, **3** and **4** between the aromatic protons of Ac–Gly–Phe and the cyclodextrin protons H-3 and H-5[a,b]

	CDProp (**1**)		CDBenz (**3**)		HMA (**4**)	
	H-3	H-5	H-3	H-5	H-3	H-5
H-*ortho*	+	+	+	+	−	+
H-*meta*	+	+	+	+	+	+
H-*para*	+	+	+	+	−	−

[a] In D_2O, $c(NaCl) = 1 \times 10^{-2}$ mol l^{-1}, $T = 300$ K; $c(\mathbf{1}, \mathbf{2}) = 1.2 \times 10^{-2}$ mol l^{-1}, $c(\mathbf{4} = 5 \times 10^{-3}$ mol $l^{-1})$, c(Ac–Gly–Phe) $= 6 \times 10^{-2}$ (for the complexes with **1** and **3**); 1×10^{-2} (for **4**).
[b] + = Intermolecular cross peak observed; − = no cross peak.

the H-3 protons. This arrangement allows interaction of the multiple positive charges of the CyD molecule with the negative *C*-terminus of the peptide.

In contrast, in the complexes with only one positive charge (**1** and **3**), no preferred inclusion geometry could be deduced (Fig. 11). *Ortho*-, *meta*- and the methylene protons from the Phe residue give cross peaks to both intracavity H-3 and H-5 protons. Both arrangements, inclusion from the primary and from the secondary side, seem to exist in these complexes.

EXPERIMENTAL

Materials. *β*-Cyclodextrin was a gift from Wacker-Chemie (Burghausen, Germany). With the exception of Ac–Gly–Phe (for preparation see below) the peptides were purchased from Bachem Biochemica (Heidelberg, Germany). CM-Sephadex C-25 was purchased from Aldrich-Chemie (Steinheim, Germany) in the Na^+ form, swollen in distilled water overnight and converted to the $NH4^+$ form by washing with 0.5 M ammonium hydro-

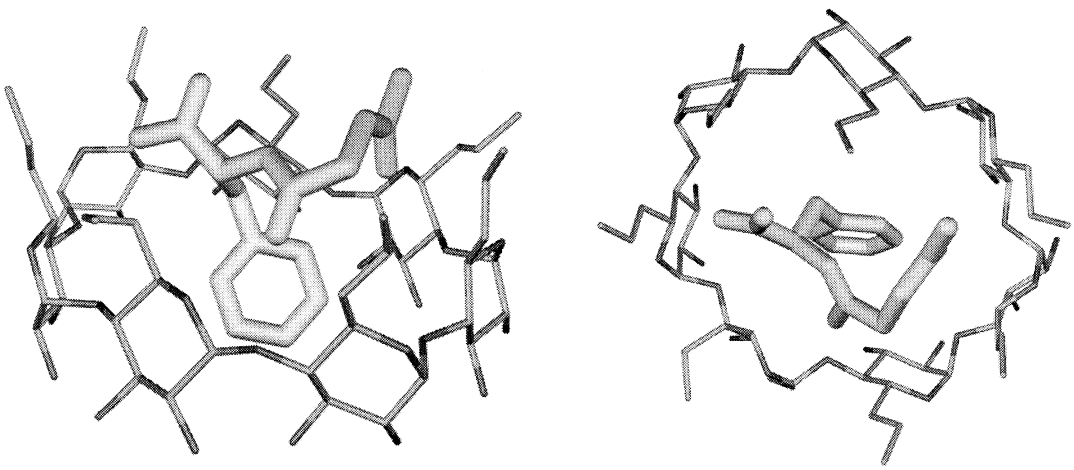

Figure 10. Complex geometry for the complex of **4** with Ac–Gly–Phe

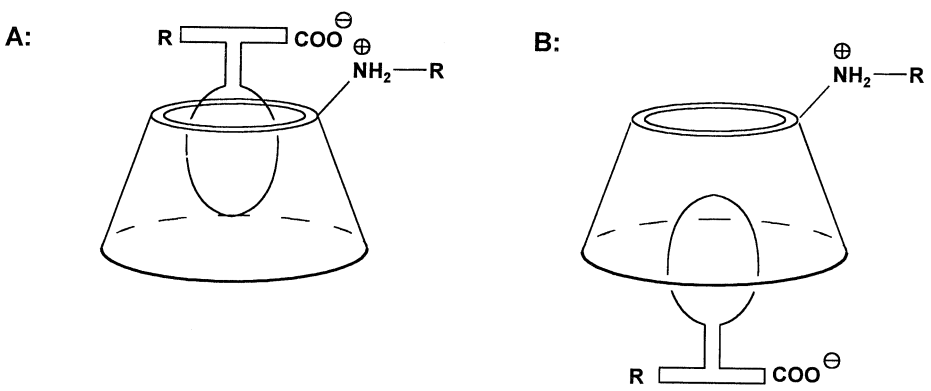

Figure 11. Schematic representation of the possible complex geometries of **1** and **3** with Ac–Ghy–Phe

gencarbonate solution followed by neutralization with water. Deuterated solvents were purchased from Deutero (Kastellaun, Germany) (deuteration 99.9%). All other chemicals were purchased from Fluka-Chemie A (Buchs, Switzerland) and used without further purification. With the exception of methylamine, all amines were distilled over KOH before use.

TLC was performed on silica-coated aluminium plates (Alugram-SIL; Macherey–Nagel, Düren, Germany) For detection the plates were treated with a solution of 5% sulfuric acid in ethanol and heated, resulting a black spot for CD-containing fractions. Elemental analysis was performed on an Elementar Analyser Modell 1106 from Carlo Elba.

Mono-6-p-toluenesulfonyl-β-cyclodextrin. To a solution of 50.0 g (44.1 mmol) of β-CD in 1.5 l of 0.4 M sodium hydroxide, 50.0 g (262 mmol) of *p*-toluene sulfonyl chloride were added with vigorous stirring. The resulting suspension was stirred for 3 h at 0 °C. The reaction mixture was filtered and neutralized with 1 M hydrochloric acid to yield a white precipitate. After filtration, the crude product was washed with acetone and dried under vacuum. Recrystallization from water gave 12.2 g (22%) of mono-6-*p*-toluenesulfonyl-β-cyclodextrin. TLC [acetic acid–chloroform–water (8:1:1, v/v)], $R_f = 0.36$.

NMR analyses confirmed the monosubstitution by the tosylate as described earlier for the same compounds.[28] NMR also showed a purity of only 92%. Repeated recrystallization from water did not lead to a improved purity of the product. The product always shows some impurities of unreacted CD tosyl chloride. As the byproducts did not lead to problems in the further preparation of the amines, no additional purification steps were necessary with the tosylate.

Monoamino-CDs 1–3. A 1.0 g (0.78 mmol) amount of mono-6-*p*-toluene sulfonyl-β-cyclodextrin was dissolved in 5 ml of the freshly distilled amine and heated at 70 °C in an atmosphere of nitrogen for 15 h. The amine was evaporated, yielding a pale-yellow syrup that was refluxed in ethanol for 30 min. The white precipitate that formed was isolated by filtration and the procedure was repeated. Purification was performed by ion-exchange chromatography on carboxymethyl-Sephadex C-25 (NH_4^+ form). The column was eluted initially with water to replace impurities. In a second step the amino-CDs bound to the column were removed by a gradient from 0 to 0.5 mol l^{-1} aqueous ammonium hydrogencarbonate. CD-containing fractions (assayed by TLC showing only one spot) were collected and dried under vaccum. The remaining white solid was dissolved three times in water and evaporated to dryness to decompose ammonium hydrogencarbonate.

Yields (after purification): **1**, 695 mg (73%); **2**, 708 mg (75%); **3**, 530 mg (42%). TLC (2-propanol–ethyl acetate–water–ammonia (7:7:5:4, v/v)], R_f = (**1**) 0.40, (**2**) 0.61 and (**3**) 0.84. Elemental analysis: **1**, calc. N 2.30, C 46.30, H 6.78, found N 2.29, C 45.05, H 6.83%; **2**, calc. N 2.30, C 46.38, H 6.62, found N 2.51, C 45.22, H 6.61%; **3**, calc. N 1.14, C 48.08, H 6.34, found N 1.08, C 44.04, H 6.41%. For NMR spectra, see NMR section.

*6-Heptakisdeoxy-6-heptakismethylamino-β-cyclodextrin (**4**).* The heptaamine **4** was synthesized in two steps from β-CD.[14] After a persubstitution of the primary hydroxy functions[15] by iodide–triphenylphoshpine, the amine was prepared with methylamine in dry ethanol and purification over carboxymethyl-Sephadex. NMR data and elemental analyses were identical with previously published data.[6]

Acetyl-glycyl-(L)-phenylalanin: Gly-(l)-Phe was protected similar to literature known synthesis.[29] Gly-(L)-Phe (1.0 g, 4.5 mmol) was dissolved in a minimum amount of a saturated aqueous sodiumhydrogencarbonate solution and acetic anhydride (0.85 ml, 9.0 mmol) added. The reaction mixture was stirred at room temperature for 10 min and refluxed for another 3 min to destroy the remaining anhydride. The reaction mixture was passed through an ion exchange column (Dowex 50W8 H^+-form). The resulting solution was evaporated to dryness yielding 686 mg (58%) of Ac–Gly–(L)–Phe as a white solid. m.p. 169 °C, TLC: (Ethylacetate, DMF 9:1) Rf = 0.44, ^{1}H NMR, DMSO-d_6, TMS internal reference, δ [ppm]: 12.82 (s, ^{1}H,) COOH, 8.18 (d, ^{3}J = 7.6 Hz, ^{1}H) NH-Phe, 8.08 (t, ^{3}J = 5.6Hz, 1H) NH-Gly, 7.32–7.21 (m, 5H) phenyl-H, 4.45 (m, 1H) CH-Phe, 3.72 (dd, ^{2}J = 16.6 Hz, ^{3}J = 5.8 Hz, 1H) CH_2a-Gly, 3.63 (dd, ^{2}J = 16.8 Hz, ^{3}J = 6.0 Hz, 1H) CH2b-Gly, 3.06 (dd ^{2}J = 13.4 Hz, ^{3}J = 4.8 Hz, 1H) CH_2a-benzyl, 2.89 (dd, ^{2}J = 13.6 Hz, ^{3}J = 8.8, 1H) CH_2b-benzyl, 1.85 (s,3H) CH3, ^{13}C-NMR, DMSO-d_6, ref = DMSO = 39.5 ppm, δ [ppm]: 172.2 (Carbox.), 169.4, 168.9 (Amide), 137.4, 129.1, 128.2, 126.4 (phenyl), 53.3 (CH), 41.7 (CH_2-Gly), 36.8 (CH_2-benzyl), 22.4 (CH_3).

Fluorescence Spectroscopy. All fluorescence measurements were carried out at 298 K in doubly distilled water using a Hitachi fluorescence spectrophotometer F-2000 and Helma 'Quarz-cells 111'. The samples were exited at 360 nm and emission intensities measured at 480, 500 and 520 nm.

NMR-Spectroscopy: NMR spectra were recorded on Bruker AM 400 (only for the routine analysis of the raw products) and on AVANCE 500 NMR spectrometers, operating at 400.1 (500.1) MHz for protons and 100.0 (125.0) MHz for carbon-13. A 5 mm dual (inverse) ^{1}H-^{13}C probehead was used at room temperature 300±K. Complexation-induced shifts (CIS) values were calculated from the difference of the shifts in the

complex and the free state using the K_A-values from the fluorescence displacement titration. (Measurement conditions see tables). 1×10^{-2} mol/l NaCl concentration was present in all solutions for an equal ion strength as in the binding constants measurements. Typical measuring conditions for the 2D spectra were: 90°–> pulses for protons and carbons, 9 and 12 s, gradient pulse length 1 ms, sweep width 4000 (2000) Hz; data size 2K/1K in the ω_2/ ω_1 direction, $\pi/3$ shifted squared sine-bell windows in both directions; delay between the scans 2 s, 8–64 scans, depending on concentration. Phase-sensitive spectra using the TPPI method were acquired. In the homonuclear experiments elimination of the water signal was always made; using in the 2D TOCSY spectra the 3–9–19 pulse sequence with gradients[16,30] and presaturation of the water signal at a power of 50 db below the maximum output in all other experiments. The 2D T-ROESY[31] spectra were acquired using for spin locking field a sequence of $180x\ 180{-}x$ pulses, each with a duration of 125 s. For a minimum sweep width in the second dimension application of quadrature detection was possible by the use of two transmitter frequencies, one for excitation in the middle of the spectrum and the other for the spin locking field at the high-field end of the spectrum. GHSQC[21] experiment was used for heteronuclear correlations. For good quality 1D TOCSY spectra the z-filtering scheme[32] with application of a selective excitation pulse was used.

Binding constants. All titrations were carried out at 298 K in water, pH 7.0, c(NaCl) = 1×10^{-2} mol l^{-1}. All binding constants are average values calculated from three emission wavelengths as mentioned above, differing by <5% from each other. In a first step the association constants between the amino-CD and the fluorescence indicator ANS were determined by a normal fluorescence titration. A CD stock solution was added to a ANS containing solution following the increase in fluorescence intensity. The results were evaluated according to a non-linear least-squares fitting procedure described earlier.[33] The K values [$K_{ass.}$ (M^{-1}) = **1**, 251; **2**, 239; **3**, 156; **4**, 2380] reflect stronger binding of the negatively charged ANS to the positively charged amino CDs in comparison with unmodified CD ($K_{ass.}$ = 110 M^{-1}), in line with earlier studies in this field.[34] In the second step stock solutions of the investigated peptides were added to an ANS–CD solution and the decrease in fluorescence intensities was followed.

The data were treated with a linear least-squares fit according to Eqn. (1),[17] yielding the peptide CD-binding constants K.

$$\frac{S_t}{P} = \frac{K_1}{K_{11}}(Q+1) \qquad (1)$$

where

$$P = \frac{S_t K_{11}}{QK_1 + K_{11}}$$

$$Q = \frac{(e - e_{1L})}{(e_1 - e)}$$

$$S = [\text{peptide}], S_t = [\text{peptide}]_{total}$$
$$L = \text{CD}, L_t = [\text{CD}]_{total}$$
$$I = \text{ANS}, I_t = [\text{ANS}]_{total}$$
$$K_1 = \frac{[IL]}{[I][L]}, K_{11} = \frac{[SL]}{[S][L]}$$

e = measured fluorescence intensity
e_I = fluorescence intensity of ANS without CD
e_{IL} = fluorescence intensity of the ANS–CD complex

with K in M^{-1}, S, P and L in M and other parameters dimensionless.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft, Bonn, and the Fonds der Chemischen Industrie, Frankfurt.

REFERENCES

1. (a) Szeijtli J, Osa T, (eds). In Comprehensive Supramolecular Chemistry, **Vol. 3**: *Cyclodextrins*. Elsevier: Oxford, 1996; (b) Duchene D, (ed). In *New Trends in Cyclodextrins and Derivatives*. Editions de Santé: Paris, 1991: 179ff; Djedaini F, Perly B. In *New Trends in Cyclodextrimes and Derivatives*. Editions de Santé: Paris, 1991; 215ff; (c) Szeijtli J. *Cyclodextrin Technology*, 1988 Kluwer: Dordrecht; (d) Wenz G. *Angew. Chem.* 1994 **33**: 803; *Angew. Chem., Int. Ed. Engl.* 1994; **106**: 851; (e) *Chem. Rev. 98* 1998; **98**: No. 5.
2. Connors K. *J.Pharm. Sci.* 1995; **84**, 843 available on-line via supplement at: http://www.pharmacy.wisc.edu/facstaff/sciences/Connors.html.
3. Connors KA, *Chem. Rev.* 1997; **97**: 1325.
4. Schneider H-J, Blatter T, Simova S. *J. Am. Chem. Soc.* 1991; **113**: 1996.
5. Schneider H-J, Hacket F, Rüdiger V, Ikeda H. *Chem. Rev.* 1998; **98**: 1755.
6. Eliseev AV, Schneider H-J. *J. Am. Chem. Soc.* 1994; **116**: 6081.
7. Hamasaki K, Usui S, Ikeda H, Ikeda T, Ueno A. *Supramol. Chem.* 1997; **8**: 125; Ikeda H, Nakamura M, Ise N, Oguma N, Nakamura A, Ikeda T, Toda F, Ueno A. *J. Am. Chem. Soc.* 1996; **118**: 10980; Usea S, Tanabe T, Ikeda H, Ueno A. *J. Mol. Struct.* 1998; **442**: 161; Hamai S, Ikeda H, Ueno A. *J. Inclus. Phenom. Mol. Recogn.* 1998; **31**: 265.
8. Narita M, Hamada F, *J. Chem. Soc., Perkin Trans.* 2000; **2**: 823.
9. Uccello-Barretta G, Balzano F, Cuzzola A, Menicagli R, Salvadori P, *Eur. J. Org. Chem.* 2000; **3**: 449.
10. Salvatierra D, Sanchez-Ruiz X, Garduno R, Cervello E, Jaime C, Vergili A, Sanchez-Ferrando F. *Tetrahedron* 2000; **56**: 3035.
11. Impellizzeri G, G Pappalardo, D'Alessandro F, Rizzarelli E,

Savioano M, Iacovino R, Benedetti E, Pedone C. *Eur. J. Org. Chem.* 2000; **6**: 1065.
12. Matsui Y, Okimoto A. *Bull. Chem. Soc. Jpn.* 1978; **51**: 3030.
13. Zhong N, Byup HS, Bittman R. *Tetrahedron Lett.* 1998; **39**: 2919.
14. Breslow R, Czarniecki MF, Emert J, Hamaguchi H, *J. Am. Chem. Soc.* 1980; **102**: 762.
15. Ashton PR, Königer R, Stoddart JF, *J. Org. Chem.* 1996; **61**: 903.
16. Hamelin B, Jullien L, Guillo F, Lehn JM, Jardy A, Robertis LDe, Driguez H. *J. Phys Chem.* 1995; **99**: 17877.
17. Connors K. *Binding Constants*. John Wiley & Sons: New York, 1987.
18. Simova S. *Magn. Reson. Chem.* 1998; **36**: 505.
19. Chen W, Fukudome M, Yuan D, Fujioka T, Mihashi K, Fujita K. *J. Chem Soc., Chem. Commun.* 2000; 541.
20. Brooks CL, Karplus M. *Methods Enzymol.* 1986; **127**: 369; Brünger AT, Karplus M. *Acc. Chem. Res.* 1991; **24**: 54.
21. Bruker Pulse Library 98/08/04, 1998.
22. Schmieder P, Zimmer S, Kessler H. *Magn. Reson. Chem.* 1991; **29**: 375.
23. Reynolds WF, Yu M, Enriquez RG, Leon I, *Magn. Reson. Chem.* 1997; **35**: 505.
24. Bonomo RP, Cucinotta V, D'Allessandro F, Impellizzeri G, Maccarrone G, Rizzarelli E, *J. Inclus. Phenom.* 1993; **15**: 167.
25. Hamasaki K, Ikeda H, Nakamura A, Ueno A, Toda F, Suzuki I, Osa T. *J. Am. Chem. Soc.* 1993; **115**: 5035.
26. Corradini R, Dossena A, Marchelli R, Panagia A, Sartor G, Saviano M, Lombardi A, Pavone V. *Chem. Eur. J.* 1996; **2**: 373.
27. Ikeda H, Nakamura M, Ise N, Toda F, Ueno A. *J. Org. Chem.* 1997; **62**: 1411.
28. Bonomo RP, Cucinotta V, D'Alessandro F, Impellizzeri G, Maccarrone G, Vecchio G, Rizzarelli E. *Inorg. Chem.* 1991; **30**: 2708.
29. Ki-Hwan K, Martin Y, Otis E, Mao J. *J. Med. Chem.* 1989; **31**: 84.
30. Sklenar V, Piotto M, Leppik R, Saudek V. *J. Magn. Reson. A* 1993; **102**: 241.
31. Hwang T-L, Shaka AJ, *J. Am. Chem. Soc.* 1992; **114**: 3157.
32. Simova S, Dimitrov V. *J. Magn. Res. A*, 1995; **117**: 292.
33. Schneider H-J, Kramer R, Simova S, Schneider U, *J. Am. Chem. Soc.* 1988; **110**: 6442.
34. Ito N, Yoshida N, Ichikawa K. *J. Chem. Soc., Perkin Trans.* 1996; **2**: 965.
35. Dean JA. *Handbook of Organic Chemistry*. 1987; McGraw Hill: New York.
36. Wang F, Sayre LS. *J. Am. Chem. Soc.* 1992; **114**: 248.
37. Chokchainarong S, Fennema OR, Connors KA. *Carbohydr. Res.* 1992; **232**: 161.

