Berberine/γ-Cyclodextrin Inclusion Structure Studied by ^{1}H-NMR Spectroscopy and Molecular-Dynamics Calculations

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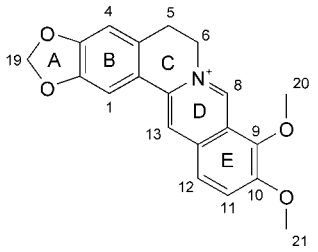
To understand the increased solubility and decreased bitter taste of berberine, a clinically important isoquinoline alkaloid, in the presence of cyclodextrins, the interaction with γ-cyclodextrin (γ-CD) in aqueous solution was studied by a combination of ^{1}H-NMR analyses and molecular-dynamics calculations. The proposed complexation mode of berberine by γ-CD could explain the increased solubility in water. No difference in germicidal activity between berberine alone and its inclusion complex with γ- or β-CD was observed, which is important to further develop the pharmacological application of berberine.

Introduction. – Berberine (= 5,6-dihydro-9,10-dimethoxy-2,3-methylenedioxyisoquino[3,2-*a*]isoquinolinium; **1**), a representative isoquinoline alkaloid with a protoberberine skeleton, is the main alkaloid of *Coptis japonica*, *C. chinensis*, *C. deltoidea*, and *C. teeta* (Ranunculaceae, rhizome), and *Phellodendron amurense* and *P. chinense* (Rutaceae, cortex), all of which are commonly used in Chinese and natural medicine. Due to its germicidal activity and intestinal regulation of noxious bacteria, **1** is being used in the preparation of a large number of gastrointestinal agents. However, its pharmacological use has two drawbacks: berberine is only slightly soluble in water and has a strong bitter taste [1].



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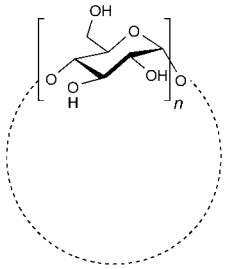

α-, β-, γ-CD (*n* = 6, 7, 8)

For quite some time now, cyclodextrins (CDs) have been recognized as useful adjuncts in pharmaceutical preparations. α-, β-, and γ-CDs, which are able to form inclusion complexes with various molecules, consist of cyclic oligosaccharides with six, seven, and eight glucose residues, respectively. The potential of CDs to form inclusion complexes with different molecules depends on the size of the cavity; the diameters of

α-, β-, and γ-CDs are *ca.* 4.5, 7.0, and 8.5 Å, respectively [2]. When considering the size of berberine (**1**), γ-CD seems to be most suitable for the formation of inclusion complexes. Interestingly, when **1** is complexed by CDs, the bitter taste of the guest decreases [3]. By identifying the inclusion geometry between **1** and γ-CD, we expect to improve our understanding at the atomic level of how both the solubility and taste of berberine can be modified to meet the requirements of modern drug-delivery systems. At present, there is no structural information available on the complex between **1** and γ-CD, although inclusion of berberine has previously been reported [4].

Here, we report the relative stabilities of two types of berberine/γ-cyclodextrin complexes and discuss their structures in aqueous solution, as studied by ^{1}H-NMR chemical-shift changes, ROESY spectral analyses, and molecular calculations. To examine how the pharmacological activity of berberine is affected γ-CD, their antibacterial and antifungal actions were evaluated by the two-fold serial broth-dilution test.

Experimental Part

General. Berberine (**1**; chloride form) and α-, β-, and γ-cyclodextrins (CDs) were purchased from *Nakarai Tesque Inc*. (Kyoto), and were recrystallized from H_2O prior to use. Solubilities were determined as follows: **1** (0.1 g) and CDs were dissolved in H_2O (3 ml) and incubated for 20 h at 40°. Then, the mixture was filtered using *Millex-HA* (*Millipore*), and its absorbance was measured at 345 nm on a *Hitachi U-2000* UV spectrophotometer.

^{1}H-NMR Experiments. Spectra were recorded on a *Varian VXR-500* apparatus at 497.2 MHz at r.t., with TSP (sodium (2-$^2H_2$, 3-$^2H_2$)-3-(trimethylsilyl)propanoate) as an external reference. Bulk susceptibility corrections to chemical shifts were not examined. Samples of 0.01 mM conc. were prepared in D_2O soln. ROESY Experiments were performed in the phase-sensitive mode, with a spin-lock mixing pulse of 200 ms.

Chemical shifts (δ) in D_2O of H-atoms of **1** as a function of the amount of γ-CD: CH_2(5) (*t*): 3.159 (**1**/γ-CD 1:0), 3.169 (1:0.3), 3.189 (1:1); 3.198 (1:1.5), 3.203 (1:3); Me(20) (*s*): 4.030 (1:0); Me(21) (*s*): 4.091 (1:0); CH_2(19) (*s*): 6.048 (1:0), 6.058 (1:0.3), 6.087 (1:1), 6.096 (1:1.5), 6.096 (1:3); H–C(4) (*s*): 6.858 (1:0), 6.857 (1:0.3), 6.849 (1:1), 6.840 (1:1.5), 6.842 (1:3); H–C(1) (*s*): 7.195 (1:0), 7.198 (1:0.3), 7.200 (1:1), 7.208 (1:1.5), 7.240 (1:3); H–C(11) (*d*, $J = 9.5$): 7.911 (1:0), 7.921 (1:0.3), 7.929 (1:1), 7.934 (1:1.5), 7.947 (1:3); H–C(12) (*d*, $J = 9.5$): 7.738 (1:0), 7.757 (1:0.3), 7.788 (1:1), 7.796 (1:1.5), 7.817 (1:3); H–C(13) (*s*): 8.166 (1:0), 8.131 (1:0.3), 8.065 (1:1), 8.027 (1:1.5), 8.058 (1:3); H–C(8): 9.599 (1:0), 9.579 (1:0.3), 9.555 (1:1), 9.550 (1:1.5), 9.553 (1:3).

Chemical shifts (δ) in D_2O of γ-CD H-atoms as a function of the amount of **1**: H–C(4): 3.598 (γ-CD/**1** 1:0), 3.600 (1:0.3), 3.601 (1:0.6), 3.602 (1:1), H–C(2): 3.658 (1:0), 3.667 (1:0.3), 3.670 (1:0.6), 3.676 (1:1); H–C(5): 3.860 (1:0), 3.849 (1:0.3), 3.850 (1:0.6), 3.840 (1:1); H_a–C(6): 3.874 (1:0); H_b–C(6): 3.874 (1:0); H–C(3): 3.939 (1:0), 3.912 (1:0.3), 3.879 (1:0.6), 3.848 (1:1); H–C(1): 5.117 (1:0), 5.116 (1:0.3), 5.114 (1:0.6), 5.113 (1:1).

Molecular-Dynamics Calculations. The three-dimensional structure of γ-CD was constructed with the *Builder* program (*Insight II/Discover-3*) [5]. The structure of berberine (**1**) was based on the X-ray crystal structure of protoberberine [6] using the *MOL/MOLIS* system [7]. According to the ^{1}H-NMR experiments, two types of inclusion complexes were considered, *i.e.*, those in which the guest enters the CD either from the side of the secondary OH groups (*Case 1*) or from that of the primary OH groups of the CD (*Case 2*). Molecular-dynamics (MD) simulations were performed by the *InsightII/Discover-3* system operation on an *SGI O2* computer. To each of the energy-minimized complexes, H_2O molecules were placed by the Monte-Carlo method within a cube cell of 25-Å diameter to examine the stability of the complex in aq. soln. A total of 428 (*Case 1*) or 432 (*Case 2*) H_2O molecules were restrained by a boundary force of 1.0 kcal mol^{-1}Å^{-1} to prevent their leakage from the system. By setting the periodic boundary condition with NVT ensemble, the MD simulations were carried out with a time step of 1 fs and a group-based nonbonded cutoff of 9.5 Å, where the CVFF [8] force field was used. The data list for nonbonded pairs was renewed every 50 time steps. The initial velocities for the respective molecular systems were given from the *Boltzmann* distribution of 10 K, and then the temp. was

elevated up to 300 K, with a relaxation time of 0.1 ps. The *Verlet* method [9] was used for the MD calculation. After the equilibration of the systems, the calculations were continued for 100 ps. Both systems reached equilibrium conditions after 80 ps, and the most-stable conditions were obtained after 94 ps for both systems within an interval of 80 to 100 ps.

Microbiological Studies. Antibacterial activity [10] against *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella enteritidis* (isolated from hospitalized patients), and *Escherichia coli* (IFO 026), as well as antifungal activity against *Candida albicans* (IFO 1061) were determined by means of the minimum-inhibitory concentration (*MIC*), using the two-fold serial broth-dilution test in liquid nutrient medium on 24-well microplates. The *MIC* value was defined as the lowest conc. of the test substance that did not induce visible growth in comparison with a blank experiment. Dilutions of the test medium from 500 to 1 μg/ml, as well as blanks, were prepared in H_2O containing 1% DMF. Several 24-well plates, in which each well contained the different conc. of sample in an appropriate growth medium, were incubated with test organisms. The 24-well plates were incubated at 35° for 24 h in the case of bacteria, and at 27° for 48 h in the case of fungi. The bacteria tested had been cultivated in 3% nutrient broth (*Nissui*, Japan) at 35°, and *C. albicans* was cultivated in 3% malt-extract powder (*Oriental*, Japan) at 27°. All experiments were run in duplicate or triplicate. For the measurement of cell growth, a const. volume of sample (200 μl) was transferred to a 96-well plate from individual wells (1 ml) of a 24-well plate. After the incubation, microbial growth was examined by measuring the optical density at 655 nm with a *Bio-Rad 450* microplate reader.

Results and Discussion. – *Solubilities of Berberine Inclusion Complexes with Cylodextrins in Water.* The solubility change of **1** in the absence and presence of CDs was measured by UV spectroscopy. When the inclusion complexes were formed, the solubility of **1** in H_2O increased *ca.* three times in the complex with α-CD, *ca.* two times with β-CD, and *ca.* 4.5 times with γ-CD (*Fig. 1*). The stability constant K of the complexes were estimated to be 14.1, 21.3, and 16.9 for α-, β-, and γ-CD, resp., based on the solubility profiles as a function of the respective CD concentrations. The solubility

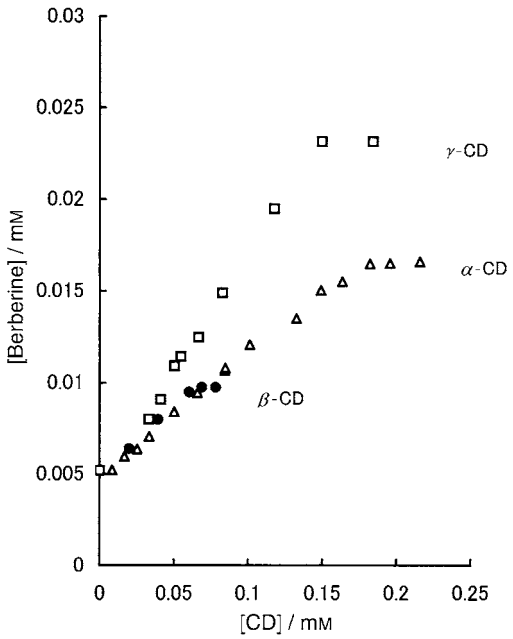

Fig. 1. *Cyclodextrin-mediated solubility changes of berberine* (**1**) *in* H_2O ($T = 40°$)

curves of *Fig. 1* show that the association constants K of the respective complexes decrease in the order γ-CD > β-CD > α-CD. Therefore, the inclusion complex with γ-CD exhibits the most advantageous increase in the solubility of **1** in H_2O.

^{1}H-NMR Analysis. The inclusion mode between **1** and γ-CD in aqueous solution was examined by ^{1}H-NMR spectroscopy at room temperature. Complexation resulted in a change of chemical shifts ($\Delta\delta_H$), occurring both in **1** and γ-CD due to steric and ring-current effects. As H–C(5) and H–C(3) of γ-CD protrude axially from the cavity, it can be expected that they are most affected by inclusion formation and that the interaction mode may be estimated from the corresponding $\Delta\delta_H$ values [11]. In addition, it has been reported that the ROESY spectra of CDs are most effective to identify H-atoms close to one another [12]. The ^{1}H-NMR chemical-shift changes $\Delta\delta_H$ of γ-CD (*Fig. 2,a*) and **1** (*Fig. 2,b*) are shown at different concentrations. The H–C(3) signal of γ-CD was most-largely shifted to higher field, and a moderate upfield shift of γ-CD H–C(5) was also observed. These high-field shifts are due to aromatic ring-current effects of berberine.

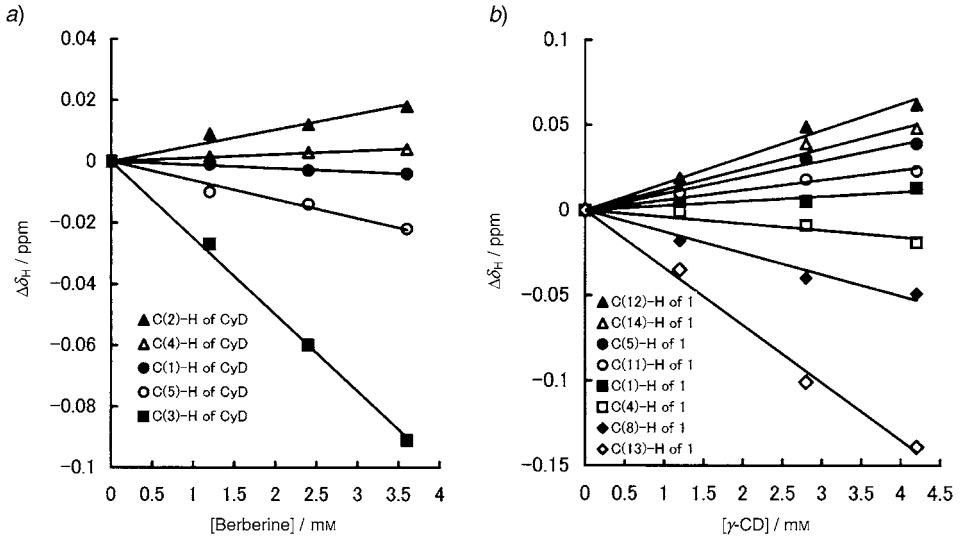

Fig. 2. *Changes in ^{1}H-NMR chemical shift ($\Delta\delta_H$) of* a) *γ-CD H-atoms as a function of berberine concentration in D_2O, and* b) *berberine H-atoms as a function of γ-CD concentration in D_2O.* The square correlation coefficients (r^2) for $\Delta\delta_H$(γ-CD) of H–C(1), H–C(2), H–C(3), H–C(4), and H–C(5) are 0.98, 0.96, 0.99, 0.99, and 0.98, resp., and those for $\Delta\delta_H$(berberine) of H–C(12), H–C(14), H–C(5), H–C(11), H–C(1), H–C(4), H–C(8), and H–C(13) are 0.98, 0.97, 0.99, 0.95, 0.90, 0.90, 0.93, and 0.99, resp.

In *Fig. 3*, a portion of the ROESY spectrum of the γ-CD/berberine complex is shown. From the cross-peaks of γ-CD-H–C(3)/**1**-H–C(1) and **1**-H–C(13); γ-CD-H–C(5)/**1**-H–C(1), **1**-H–C(4), and **1**-H–C(14); and γ-CD-H–C(6)/**1**-H–C(14) pairs, it is obvious that the B-ring of **1** is located near H–C(3) and H–C(5) of γ-CD.

Molecular-Dynamics Calculation. The stability of the **1**/γ-CD complex was estimated by MD simulations. From the ^{1}H-NMR experiments, two types of inclusion complexes were considered: the case in which the A-ring of the guest inserts from the face of the secondary OH groups (*Case 1*), and the case in which it inserts from the side

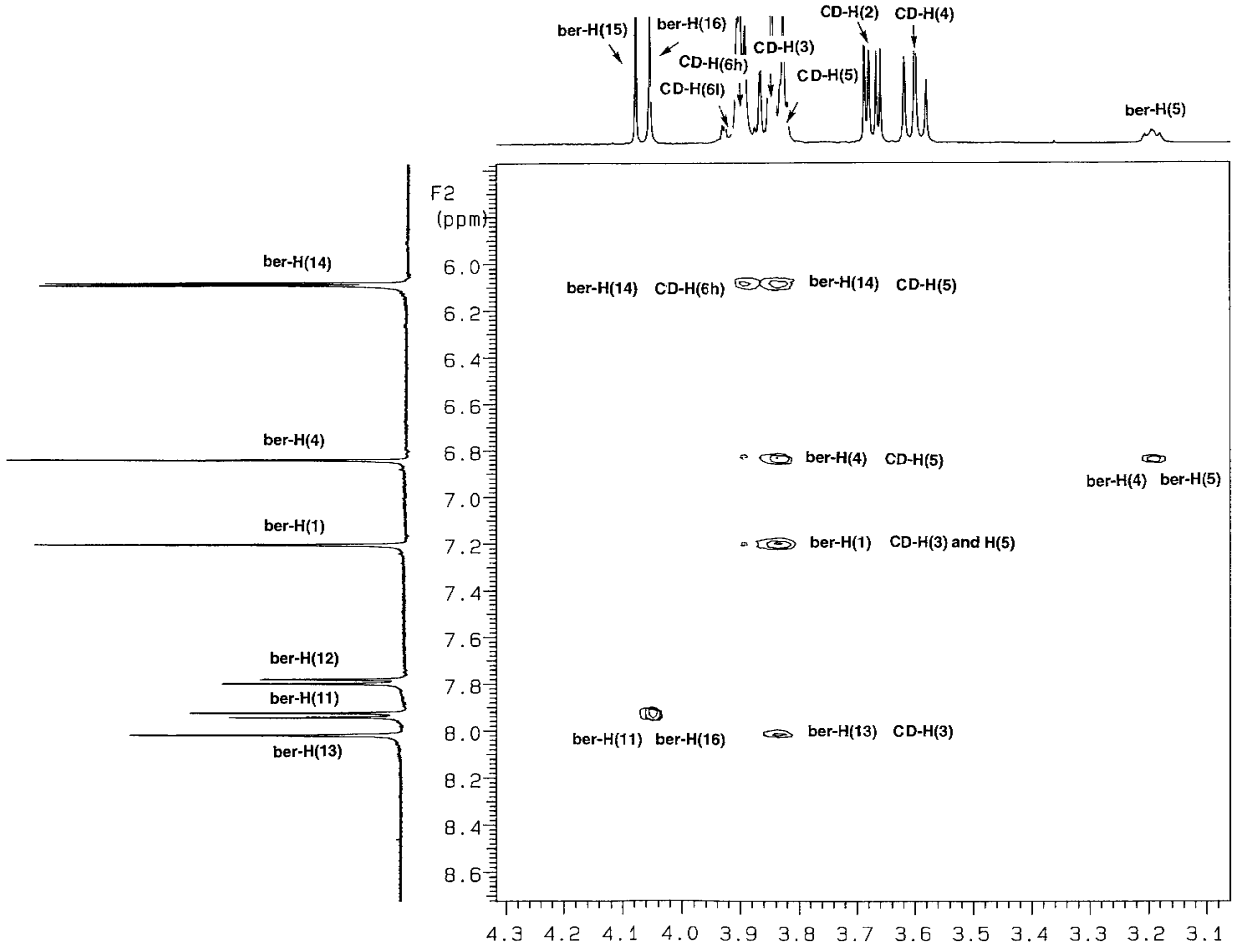

Fig. 3. *Part of the 500-MHz ^{1}H-NMR spectrum of berberine* (10 mM) *in D_2O in the presence of an equimolar amount of γ-CD* ($T = 298°$)

of the primary OH groups of γ-CD (*Case 2*). The initial geometry and a snapshot (at 94 ps simulation) of both cases are shown in *Fig. 4*. The D- (*Case 1*) or C-ring (*Case 2*) of berberine (**1**) was locked at the entrance of γ-CD, without reaching the inside of the cavity during the simulation. The MeO O-atom at C(9) of **1** formed an intermolecular H-bond to the C(1)–OH group of γ-CD (O···O 3.58 Å) in *Case 1*. This H-bond, together with hydrophobic interactions, seems to stabilize this particular inclusion geometry.

The results of the 100-ps MD simulations are summarized in *Table 1*. In *Case 1*, the initial structure of γ-CD changed upon incorporation of the guest molecule, *i.e.*, γ-CD was distorted with a long stem of 17.86–19.57 Å and a short stem of 7.23–6.22 Å. In addition, the molecular movement of **1** was different between *Cases 1* and *2*. In the former, the averaged root-mean-square deviation (rmsd) of **1** was 1.556 Å, and its rotation toward the cavity of γ-CD was *ca.* 80°. In *Case 2*, however, no rotation occurred (rmsd = 0.535 Å). This difference is presumably due to H-bond formation between the C(1)–OH group of γ-CD and O–C(9) of **1**, as stated above. As judged from the total and potential energies, the inclusion complex of *Case 1* could be more stable than that of *Case 2*.

Table 1. *Results of the Molecular-Dynamics Simulations for Two Types of 1 : 1 Inclusion Complexes* (see text and *Fig. 4*) *between Berberine* (**1**) *and γ-Cyclodextrin in H_2O* (rmsd = root mean-square difference)

	Case 1	*Case 2*
Average total energy [kcal/mol]	– 3418	– 3335
Average potential energy [kcal/mol]	– 4749	– 4679
Rmsd of γ-CD [Å/(336 atoms)]	1.924	1.810
Rmsd for **1** [Å/(86 atoms)]	1.556	0.535
Long stem [Å] of γ-CD before simulation	17.86	14.49
Long stem [Å] of γ-CD after simulation	19.57	14.88
Short stem [Å] of γ-CD before simulation	7.23	9.62
Short stem [Å] of γ-CD after simulation	6.22	6.38

Antibacterial and Antifungal Activities of Berberine/CD Complexes. Berberine (**1**) has antibacterial and antifungal properties [1][10]. The question was whether this activity is affected in the inclusion complexes with CDs. We determined the germicidal activities of **1**/γ-CD and **1**/β-CD complexes against *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella enteritidis*, *Escherichia coli*, and *Candida albicans*, based on the minimum-inhibitory concentration (*MIC*) determined by means of the twofold serial broth-dilution test (*Table 2*). Berberine was effective against *Staphylococcus aureus*, *Salmonella enteritidis*, and *Candida albicans*, and no difference was observed in germicidal activity between **1** alone and its γ- or β-CD complexes.

Table 2. *Antimicrobial Activities in Terms of Minimum-Inhibitory Concentrations* (*MIC* [μg/ml]) *of Berberine* (**1**) *and its 1 : 1 β- and γ-Cyclodextrin Complexes in H_2O*

	S. aureus	*B. subtilis*	*E. coli*	*S. enteritidis*	*C. albicans*
1	500	> 500	> 500	250	250
1/β-CD	500	> 500	> 500	250	500
1/γ-CD	500	> 500	> 500	250	250

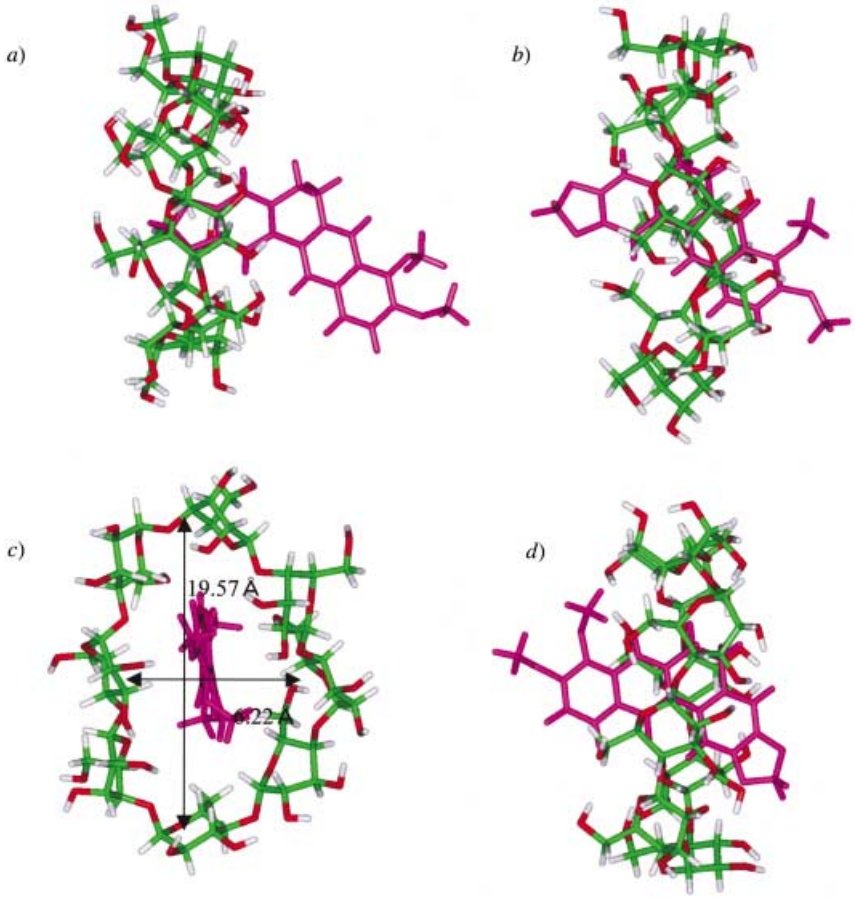

Fig. 4. *Calculated three-dimensional structures of 1:1 berberine/γ-cyclodextrin complexes.* a) *Initial structure at the beginning of the simulation*. b) *Side and* c) *top views of the most-stable complex* (*Case 1*; snapshot at 94-ps simulation time). d) *Side view of the second-most-stable complex* (*Case 2*; snapshot at 94-ps simulation time). The different modes of insertion are best seen in b) *vs.* d). In *Cases 1* and *2*, the A-ring of **1** protrudes from the face of the primary and secondary OH groups of γ-CD, resp.

Conclusions. – ^{1}H-NMR analysis and molecular-dynamics calculations of the inclusion complex between berberine (**1**) and γ-cyclodextrin (CD) in H_2O indicated an inclusion geometry in which rigid **1** is fully wrapped by the γ-CD molecule. As a result, the solubility of **1** in H_2O is considerably increased by γ-CD, but its germicidal activity is little affected by the inclusion. The increase in solubility of **1** can be largely rationalized by hydrophobic effects (reduction of total solvent-accessible hydrophobic surface). Our results indicate that the application of γ-CD inclusion complexes is very useful for the delivery of hydrophobic alkaloidal drugs.

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