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ORIGINAL ARTICLE

Host–guest complexation of antioxidative caffeic and ferulic acid amides with a functionalized cyclophane

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Abstract Host−guest complexation has been studied by ^{1}H NMR on the benzyl and phenethyl amides of ferulic and caffeic acids as the guests in chloroform and acetonitrile; the counter host is a cyclophane which integrates four phenylene rings, amino and amide groups in the macrocyclic framework and bears four pendant methyl acetate ester arms. CAPE, one of the best known natural antioxidants, also has been studied for comparison. Among the guests studied, ferulic acid benzyl amide shows NMR shifts due to the formation of a host−guest complex in chloroform. The complexation occurs in two steps with the formation constants $K_1 = [HG]/[H][G] = 6\ M^{-1}$ and $\beta_2 = [HG_2]/[H][G]^2 = 87\ M^{-2}$. Two guest molecules are bound on the surface of the macrocyclic framework of a host molecule by two hydrogen bonds, NH(host amide)···O=C(guest amide) and C=O(host ester)···HO(guest phenol). The latter hydrogen bond may protect the bioactive site, i.e., phenol OH, of guest molecules captured in the complex against undesirable oxidation. This feature is observed only for ferulic acid benzyl amide in chloroform; the cyclophane ester interacts with this amide, distinctively from the other hydroxycinnamic acid derivatives.

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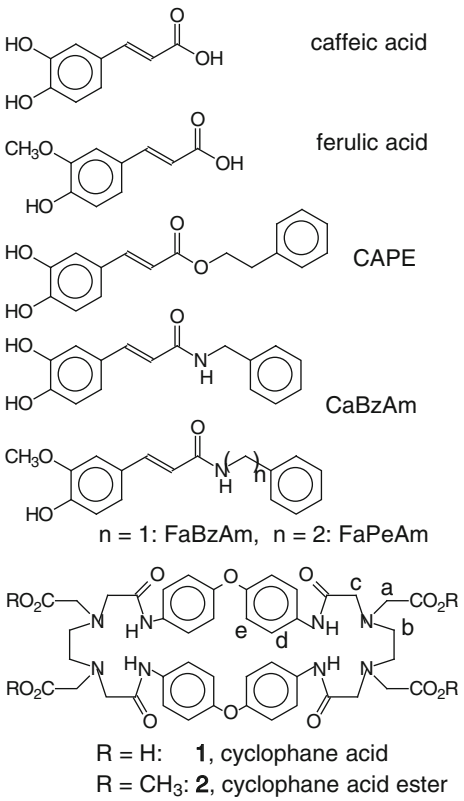
Introduction

Caffeic and ferulic acids (Scheme 1) and their esters belong to the family of hydroxycinnamic acid derivatives distributed widely in the plant kingdom, and are potential natural antioxidants that could prevent oxidative rancidity in foods and oxidative damages in vivo relating to diseases such as cancer, diabetes, and cardiovascular, Alzheimer's, and Parkinson's diseases [1–12]. Our exploration into antibacterial and antifungal properties in foodstuff has found the antibacterial activity of caffeic acid phenethyl ester (well-known with abbreviation CAPE) on some strains of *Staphylococcus aureus* [13, 14]. Further, we have studied the potential application of CAPE in the control of *Alternaria alternate*, fungus that causes major economic losses in fruit and vegetables harvested [15]. Since the ester group is metabolically labile [16, 17], the amide derivatives of caffeic acid have been synthesized, which have high antioxidative capacity as well [18]. Notwithstanding particular attention on the bioactivities, these natural and synthetic hydroxycinnamic acid derivatives have disadvantages related to the insolubility in water and the bare solubility in organic solvents of low polarity.

Some of unfavorable properties, such as low solubility and chemical instability, of a substance are expected to be improved by construction of its host−guest complexes with appropriate receptors; even new functions such as molecular-sensing and controlled release of a drug may be developed. Cyclodextrins and functionalized cyclophanes are typical hosts that can construct a variety of complexes in which a host molecule binds a guest molecule of



Scheme 1 The guests: caffeic and ferulic acid derivatives (above). The host: cyclophane acid ester 2 (below)

appropriate polarity and dimension within the hydrophobic cavity or on the hydrophilic exterior surface [19–25]. Regarding hydroxycinnamic acid esters and amides, however, a report on their host−guest complexes has not been found in our bibliographic search; only caffeic acid has been reported to form an inclusion complex with β-cyclodextrin [26]; as other polyphenol guests, some flavonoids have been found to be encapsulated by β-cyclodextrin, and the possible improvement of the pharmaceutical functions has been pointed out [27]. This fact has prompted us to search for cyclophane-type hosts capable of binding hydroxycinnamic acid derivatives in non-aqueous media. Among host−guest binding forces, hydrogen bonding is predominant in organic solvents [28]. From this viewpoint, our special interest has been directed to the complexation of the amide derivatives, because amide group is capable of forming strong hydrogen bonds to assemble supramolecules and play important roles in biological systems.

Previously, we have synthesized cyclophanes functionalized with amide group and pendant carboxyl group, and have found that these "azacyclophane acids" form inclusion complexes with bioactive amines such as dopamine, histamine and tryptamine in aqueous media [29–31]. A representative is cyclophane acid **1** shown in Scheme 1. This cyclophane acid is insoluble in organic solvents, but its methyl ester (**2**) in Scheme 1) is soluble in common organic solvents to bind simple organic acids with hydrogen bonding at the amide group in chloroform [32]. The cyclophane acid ester is, therefore, expected to function as a receptor toward the amide derivatives of caffeic and ferulic acids through multi-point hydrogen bonding, on the basis of the mutual sizes of the host and guest molecules. The present paper reports that, among the target guests shown in Scheme 1 with abbreviations, ferulic acid benzyl amide (FaBzAm) shows clear evidence for the formation of a host−guest complex in which two FaBzAm molecules are bound on the surface of the macrocyclic framework of a cyclophane ester molecule.

Experimental

Cyclophane acid ester **2** was prepared by esterification of cyclophane acid **1** with iodomethane [32–34]; the cyclophane acid was synthesized by a reaction between ethylenediaminetetraacetic (EDTA) dianhydride and bis(4-aminophenyl) ether [29], and dried in vacuum at 80 °C for 8 h before use. The amide-based guests, CaBzAm, FaBzAm and FaPeAm, were prepared from the appropriate acids and amines by using benzotriazol-1-yl-oxytris(dimet-hylamino)phosphonium hexafluorophosphate (BOP) as a coupling agent, and purified on a silica gel column [18]. CAPE was synthesized from caffeic acid and phenethyl alcohol in the presence of *p*-toluene sulfonic acid as a catalyst [35]. The formation and purity of all substances were confirmed by ^{1}H NMR.

NMR spectra were obtained with a Varian Mercury 300 spectrometer operating at 300 MHz at a temperature of 30 °C; the internal reference was TMS. The complexation was studied in $CHCl_3$-*d* (99.9% atom D) and CH_3CN-d_3 (99.8% atom D); both solvents were supplied from Aldrich, and were dried over molecular sieve when necessary. Titration of FaBzAm was performed by changing the total concentration of the guest $[G]_t$ from 20 to 160 mM (mM = mmol dm^{-3}) at the constant total concentration of the host $[H]_t$ of 5 mM in $CHCl_3$-*d*; for the majority of other host − guest systems, $[H]_t$ was 2.5 mM, and $[G]_t$ from 10 to 80 mM because of the low solubility.

Results and discussion

NMR titrations of the host−guest systems

The caffeic acid derivatives are soluble enough for NMR titration only in acetonitrile among common deuterated solvents, and the ferulic acid derivatives are soluble in

chloroform; only FaBzAm is soluble in both solvents. Titrations were carried out in the appropriate solvents by changing the total concentration of the guests $[G]_t$ while the total concentration of the macrocyclic host $[H]_t$ was kept constant. Table 1 shows the shifts Δ_H of host protons with reference to δ in the absence of a guest at selected $[H]_t$ and $[G]_t$: $\Delta_H = \delta_H([G]_t) - \delta_H(0)$ where $\delta_H([G]_t)$ is the chemical shift δ_H of a host proton at a guest concentration of $[G]_t$, and $\delta_H(0)$ is δ_H at $[G]_t = 0$. Among the guests studied, FaBzAm causes significant changes in δ_H in $CHCl_3$-*d*, and plots of $\Delta_H([G]_t)$ against $[G]_t$ give titration curves characteristic of complex formation as shown in Fig. 1. Other amide guests cause very small changes in δ_H. Even FaBzAm little influences δ_H in CH_3CN-d_3 indicating a solvent effect (Table 1). The study of complex formation has been extended to CAPE for comparison, because it is the most-studied antioxidant among hydroxycinnamic acid derivatives. Although the amide NH of the host exhibits a considerable NMR shift in the presence of CAPE, the shifts of the CH protons were too small for concluding complex formation (Table 1). Thus, only FaBzAm in chloroform provides convincing evidence for complex formation. The present work, therefore, has focused on this host−guest complexation.

Formulation and determination of formation constants

The host molecule is composed of two equivalent moieties whose size is comparable to that of a guest molecule. Potentially, therefore, each host molecule is capable of binding up to two guest molecules to yield an HG_2-type complex:

$$H + G \rightleftharpoons HG \quad (1)$$

$$HG + G \rightleftharpoons HG_2 \quad (2)$$

The formation constant of each step and the overall constant of HG_2 are defined by:

$$K_1 = [HG]/[H][G] \quad (3)$$

$$K_2 = [HG_2]/[HG][G] \quad (4)$$

$$\beta_2 = [HG_2]/[H][G]^2 = K_1 \cdot K_2 \quad (5)$$

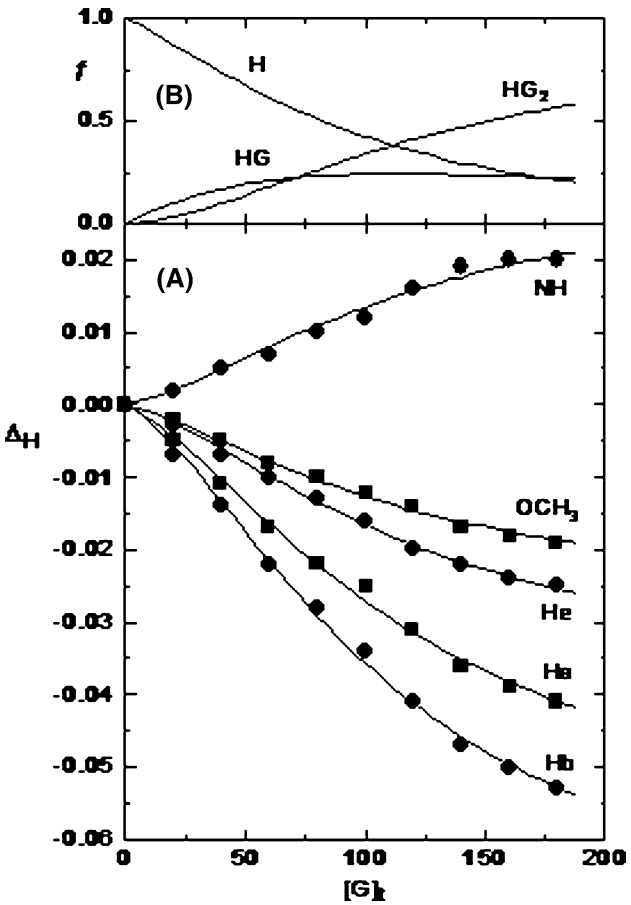


Fig. 1 *a* Changes in ^1H NMR δ of the cyclophane ester host in the presence of guest FaBzAm in $CHCl_3$-*d* at 30 °C: $\Delta_H = \delta_H([G]_t) - \delta_H(0)$ as a function of guest concentration $[G]_t$ (in 10^{-3} M) at the constant host concentration $[H]_t$ 5×10^{-3} M. The solid lines show the best fits based on the model of two-step complex formation of HG_2. *b* Mol fractions *f* calculated for H, HG and HG_2 species against $[H]_t$ (5×10^{-3} M) on the basis of the formation constants $K_1 = 6.0\ M^{-1}$ and $K_2 = 16.6\ M^{-1}$ determined from the Δ_H of proton b

Since a single ^1H NMR signal is observed for every pair of equivalent protons throughout the titrations, the reaction equilibrium is rapid compared with the NMR observation time scale. In such a fast exchange case, the chemical shift of a proton is given by an average of the values intrinsic of species that coexist in a reaction, as follows:

Table 1 ^1H NMR shifts of the cyclophane ester host (**2**) in the presence of caffeic and ferulic acid derivatives as guests at a given total concentration (in 10^{-3} M) of the host $[H]_t$ and that of a guest $[G]_t$; shift of proton H(*n*) is given with reference to δ in the absence of a guest, $\Delta_H = \delta_H([G]_t) - \delta_H(0)$ (for labeling proton *n* and the abbreviation of the guests see Scheme 1); $T = 30$ °C

Guest	Solvent	$[H]_t$:$[G]_t$	NH	CH_2(a)	CH_2(b)	CH_2(c)	ArH(d)	ArH(e)	OCH_3
FaBzAm	$CDCl_3$	5.0:80	0.010	–0.022	–0.028	–0.031	–0.009	–0.013	–0.010
	CD_3CN	2.5:80	0.010	–0.005	–0.009	–0.001	–0.001	–0.007	–0.003
FaPeAm	$CDCl_3$	2.5:80	0.013	–0.007	–0.011	–0.007	–0.002	–0.005	–0.006
CaBzAm	CD_3CN	2.5:50	0.010	–0.002	–0.005	0.004	–0.004	–0.005	–0.002
CAPE	CD_3CN	2.5:80	0.025	0.000	–0.003	0.015	–0.001	–0.004	–0.002

$$\Delta_{Hj}\left([G]_{tj}\right) = \left(\Delta_{HC1}[HG]_j + \Delta_{HC2}[HG_2]_j\right)/[H]_t \quad (6)$$

Here, the subscript j stands for the jth sample solution of a total concentration of guest $[G]_{tj}$, Δ_{HC1} is the chemical shift change Δ_H of the host in complex HG with reference to δ_H of the free host, and Δ_{HC2} is that in complex HG_2: $\Delta_{HC1} = \delta_H(HG) - \delta_H(H)$, and $\Delta_{HC2} = \delta_H(HG_2) - \delta_H(H)$, where $\delta_H(H)$ is equated to δ_H at $[G]_{tj} = 0$. On the basis of mass balances, $[H]_t = [H] + [HG] + [HG_2]$ and $[G]_t = [G] + [HG] + 2[HG_2]$, Eqs. 3 and 4 lead to the following cubic equation of [G].

$$[G]^3 + \left(1/K_2 + 2[H]_t - [G]_t\right)[G]^2 + \left(1/\beta_2 + [H]_t/K_2 - [G]_t/K_2\right)[G] - [G]_t/\beta_2 = 0 \quad (7)$$

For given K_1 and K_2, the cubic equation is solved numerically, providing [H], [HG] and $[HG_2]$ successively, and then the spectral change Δ_{Hj} is calculated by Eq. 6 for assumed Δ_{HC1} and Δ_{HC2} values. A set of four parameters, K_1, K_2, Δ_{HC1} and Δ_{HC2}, that reproduces best an observed titration curve has been determined by a non-linear least-squares fitting [36]. The Δ_H values caused by FaBzAm in $CHCl_3$-d are large enough for determination of the formation constants, and the titration curves are reproduced well with parameters given in Table 2 as shown in Fig. 1. The formation constants determined from the different protons agree with one another in a deviation of about 10%; the means are $< K_1 > = 6.0 \pm 0.7$ and $< K_2 > = 14.7 \pm 2.0$. When only a 1:1 complex HG is assumed to form, the formation constant K and the shift $\Delta_{Hj}([G]_t)$ are readily formulated [29]. Although the resulting $\Delta_{Hj}([G]_t)$ reproduced each of observed titration curves with a tolerable deviation, the fitness between the shapes of the observed and calculated titration curves was poor compared with that for the two-step complex formation. In addition, the constants K determined for different protons showed a deviation as large as 100% from one another. These facts suggest that the successive formation of two species HG and HG_2 is reasonable rather than the formation of a single species HG.

Binding sites in the complex

The major binding forces of host–guest complexes in organic solvents originate from hydrogen bonding [28]. One of the most convincing evidences for hydrogen bonding is given by the down-field NMR shift (or an increase in δ) of the participating acidic proton [32, 37]. The amide NH signal of the host shows an increase in Δ_H with increasing the concentration of FaBzAm (Fig. 1 and Table 1). This observation indicates the participation of the amide proton in the hydrogen bonding. The most probable counter H-bonding site in the guests is amide C=O to form NH(host)···O=C(guest) binding. This hydrogen bond has been confirmed by ^{1}H NMR of FaBzAm in the presence of the host; Table 3 represents changes in δ, $\Delta_G = \delta_G([H]_t) - \delta_G(0)$, at selected concentrations $[G]_t$ and $[H]_t$ in $CHCl_3$-d. The significant positive Δ_G value observed for CH(2) of FaBzAm supports the formation of NH(host)···O=C(guest), because the hydrogen bond lowers electron density in the conjugated system O=C–CH(2)= in the guest. Potential H-donating sites in FaBzAm include phenol OH and amide NH. Evidence for hydrogen bonding (i.e., positive Δ_G) is found for the OH proton; by contrast, the amide NH proton shifts toward a direction opposite to that predicted for hydrogen bonding (Table 3). The hydrogen bonding at the OH group is consistent with the negative Δ_G values of H(5, 6, 9) of the o-methoxyphenol ring on which electron density is increased as a result of partial proton donation of phenol OH to an electron donor atom in the host. The counter H-accepting site in the host is either amino nitrogen or ester C=O oxygen. Hydrogen bonding at the amino nitrogen would lead to an increase in δ of the adjacent protons NCH_2(a, b, c) [38]. To the contrary, these protons

Table 2 Formation constants K_n and β_2 determined from ^{1}H NMR shifts δ of cyclophane protons for HG and HG_2 complexes between the cyclophane ester host (**2**) and guest FaBzAm, and the shifts, Δ_{HCn}, of the complexes, with reference to the free cyclophane: $K_1/M^{-1} = [HG]/[H][G]$; $K_2/M^{-1} = [HG_2]/[HG][G]$; $\beta_2/M^{-2} = [HG_2]/[H][G]^2$; Δ_{HC1} is $\delta_H([G]t = \infty) - \delta_H(0)$ for complex HG, and Δ_{HC2} that for HG_2. Solvent, $CHCl_3$-d; $T = 30$ °C

Proton	K_1	K_2	β_2	Δ_{HC1}	Δ_{HC2}
NH	5.1	16.8	85	0.013	0.031
CH_2(a)	5.5	13.9	76	−0.031	−0.063
CH_2(b)	6.0	16.6	100	−0.030	−0.078
CH_2(c)	6.3	15.5	97	−0.036	−0.083
ArH(e)	7.1	13.1	93	−0.012	−0.040
OCH_3	6.0	12.0	72	−0.014	−0.030

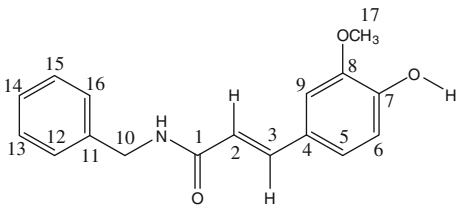
The averages over the values of different protons: $< K_1 > = 6.0 \pm 0.7$; $< K_2 > = 14.7 \pm 2.0$; $< \beta_2 > = 87 \pm 11$. The uncertainty of each formation constant is in the range 5–10%

show decreases in δ upon complex formation (Fig. 1 and Table 1). When the ester C=O of a host molecule forms a hydrogen bond with the phenol OH of a guest molecule, the *o*-methoxyphenol ring of the guest can orient in such a way that its ring plane faces the CH_2(a, b, c) groups of the host. In such a structure, the ring-current field induced by the methoxyphenol ring can result in $\Delta_H \sim -0.02$ to -0.03 observed for protons H(a, b, c), because the ring-current shift caused by a benzene ring may amount up to -2.2 ppm for a resonant proton that resides above the aromatic ring plane in a distance of a van der Waals contact 2.9 Å [39]. Thus, the NMR shifts of CH_2(a, b, c) protons supports hydrogen bonding at the ester C=O of the host. All of the observed NMR shifts consistently indicate that the host−guest assembly is constructed by two-point hydrogen bonding composed of NH(host amide)···O=C(guest amide) and C=O(host ester)···HO(guest phenol). A possible molecular arrangement involving these hydrogen bonds is visualized in Fig. 2: two guest molecules are located on the opposite hemispheres of the host molecule for steric reasons, and the methoxyphenol ring plane faces CH_2(a) and CH_2(b) groups. In acetonitrile, Δ_H values are very small compared with those in chloroform except that the amide proton shifts are comparable. Probably, the high polarity of acetonitrile weakens hydrogen bonding between the host and the guest, and alters relative molecular orientations.

As CAPE causes very small shifts Δ_H of the host, the changes in δ_G of the guests are not significant either; the Δ_G value of every proton of CAPE was of the order of 0.001 at $[G]_t$ 4 mM and $[H]_t$ 32 mM. In addition, the signal of phenol OH of the guest was undetectable due to extreme

Table 3 ^{1}H NMR shifts of proton H(*n*) of FaBzAm in the presence of the cyclophane ester host: the shifts are referenced to the corresponding δ_G of the free guest, $\Delta_G = \delta_G([H]_t) - \delta_G(0)$; solvent, $CHCl_3$-*d*; $[G]_t$, 4×10^{-3} M; $[H]_t$, 32×10^{-3} M; *T*, 30 °C

Proton	Δ_G	Proton	Δ_G
Amide NH	–0.010	OH	0.214
CH(2)	0.019	H(5)	–0.019
CH(3)	–0.010	H(6)	–0.017
CH_2(10)	–0.012	H(9)	–0.007
Phenyl H	–0.008 to 0.012	OCH_3(17)	–0.023



FaBzAm

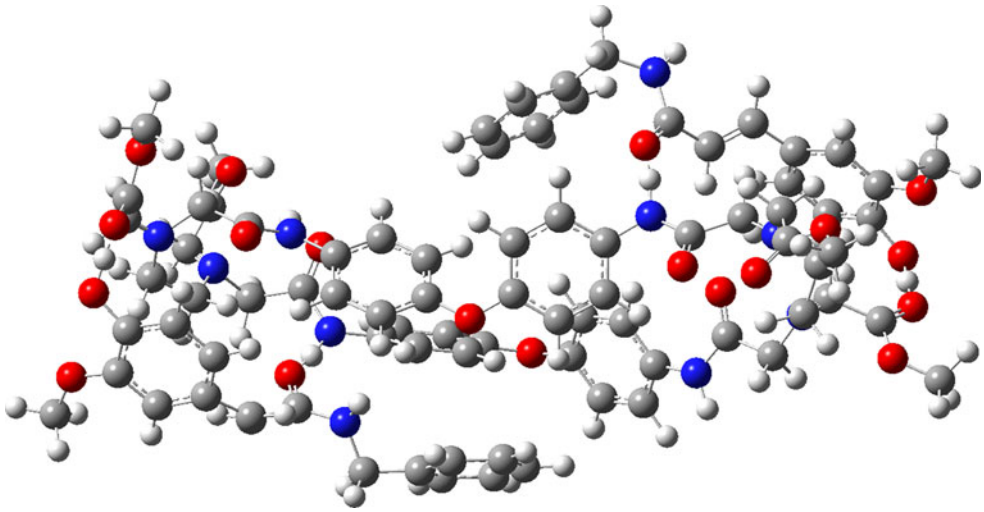


Fig. 2 View of host−guest interaction concluded from the ^{1}H NMR shifts of FaBzAm (guest) and cyclophane acid ester (host) in their complex: the structure is drawn just to visualize (1) two-point hydrogen bonding, NH(host amide)···O=C(guest amide) and C=O(host ester)···HO(guest phenol), and (2) the location of CH_2(a) and CH_2(b) of the host above the *o*-methoxyphenol ring plane of the guest, with the aid of molecular mechanics MM + on HyperChem (Professional Version Rel. 6.03, Hypercube Inc., Gainesville, FL, USA)

line-broadening. No convincing evidence for complex formation has been obtained from δ_G of the guest either.

Complexation properties of the host

In the FaBzAm−cyclophane complex, the absolute value $|\Delta_{HC1}|$ of the HG species is approximately half the $|\Delta_{HC2}|$ value of the HG_2 species (Table 2). The complexation equilibrium and the accompanying molecular reorientation occur rapidly compared with the NMR time scale, because every proton in the host as well as in the guests exhibits a single ^{1}H NMR signal throughout the titrations. In such a fast-exchange case, the ratio of Δ_{HC1} and Δ_{HC2} is correlated to the probability that each binding site in a host molecule forms a hydrogen bond with a guest site. A binding probability in the HG species is supposed to be approximately half that of the corresponding HG_2 species if both species have identical strength of local binding forces. This is the case because of $2|\Delta_{HC1}| \approx |\Delta_{HC2}|$. Upon the formation of an HG complex, the molecular conformation of the host may be rearranged so as to facilitate complexation with a second molecule. As a result, the value of K_2 is much large than K_1, but local binding forces are unchanged in complexation steps.

The parent cyclophane acid (**1**) forms a 1:1 inclusion complex with phenethylamine and dopamine in aqueous media due to hydrophobic effect [29]. In contrast, hydrophobic interaction is ineffective in organic solvents, and hydrogen bonding is dominant to allow the cyclophane ester (**2**) to capture two guest molecules on the surface of the macrocyclic framework because of fitness between the arrangements of the hydrogen bonding sites in the reactants.

Conclusion

NMR studies have given evidence for formation of a host−guest complex between ferulic acid benzyl amide and cyclophane ester **2** in chloroform. The major species is HG_2, which is stabilized by two-point hydrogen bonding composed of NH(host amide)···O=C(guest amide) and C=O(host ester)···HO(guest phenol). Since the phenol OH is responsible for the bioactivities [12], the hydrogen bond C=O(host ester)···HO(guest phenol) is supposed to suppress the antioxidative and free-radical scavenging activities of captured guest molecules or to protect the bioactive site against undesirable oxidation. This feature is notable in connection with the use of a receptor in controlled-release of a bioactive substance, and it is appreciable merely for FaBzAm in chloroform. A measure for the capacity of controlled-release is the concentration $[G]_{t,\ 1/2}$ at which half of guest molecules are captured by host molecules, or $[G] = [G]_{t/2}$, under $[G]_t = [H]_t$. The stability ($\sim 90\ M^{-2}$) of the present host−guest system gives a $[G]_{t,\ 1/2}$ value of about 0.1 M (or ~3% (w/w)). This concentration is too high for practical use. Still the present study has shown an example for receptors capable of capturing a ferulic acid derivative in a protective manner in lipophilic media.

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