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Cyclodextrins as carriers for kavalactones in aqueous media: Spectroscopic characterization of (*S*)-7,8-dihydrokavain and β-cyclodextrin inclusion complex

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ABSTRACT

Kavalactones represent the active constituents of kava–kava (*Piper methysticum* G. Forster), endowed with sedative and anaesthetic properties. Kavalactones are polar constituents, but poorly soluble in water with a low bioavailability. In this study, the formation of inclusion complexes of one of the most representative kavalactone isolated from kava–kava extract, (*S*)-7,8-dihydrokavain (DHK), with β-cyclodextrin (β-CyD) was investigated mainly by spectroscopic methods. NMR experiments were extensively used for the complete characterization of the complex and included ^{1}H NMR complexation shifts analysis, ^{1}H NMR diffusion measurements (DOSY), and ROESY experiments. In particular DOSY experiments demonstrated that in the presence of β-CyD the translational diffusion of kavalactone is sizably slowed down (2.5×10^{-10} m^2/s) with respect to the free drug (4.4×10^{-10} m^2/s) according to the inclusion of DHK in the cavity of (β-CyD). ROESY experiments confirmed the inclusion of DHK in the hydrophobic pocket of β-CyD through the primary hydroxyl rim, being the most relevant interactions between the H3′ of β-CyD and the *ortho* protons on the phenyl ring of the DHK, and between H5′ of β-CyD and the *meta*/*para* protons of DHK phenyl ring. The inclusion of the phenyl ring of DHK, leaving the lactone moiety outside of CyD was also confirmed by the induced CD effects. The binary solution DHK/β-CyD shows a 50% intensity increase of the negative band of the π–π* transitions of the phenyl ring with respect to the absorption observed with DHK alone. Molecular dynamics simulations results corroborated and further clarify observed spectroscopic data. It was found that the phenylethyl substituent at C6 has a preferential equatorial position in the free state, and an axial one in the complex, justifying the large downfield shift experienced by H6 of DHK upon binding. Finally the influence of β-CyD on water solubility of DHK was investigated by phase-solubility studies. In the range 2–4 mM of host, solubility of DHK was increased only two-fold, but being β-CyD also a penetration enhancer, *in vivo* studies will be performed to clarify a possible role of the complex on the bioavailability of DHK.

1. Introduction

Kava–kava (*Piper methysticum* G. Forster) is an herbal drug traditionally used in social and ceremonial life of Pacific islands inhabitants from ancient times for the soporific and narcotic effects. Kavalactones (kavapyrones) represent the active constituents of kava–kava, endowed with sedative and anaesthetic properties [1,2]. Kavalactones are polar constituents, but poorly soluble in water and for this reason the traditional kava–kava beverage was prepared with coconut milk [1]. In order to develop an innovative formulation whit increased bioavailability, for local anaesthesia, the enantiopure (*S*)-7,8-dihydrokavain (DHK see Fig. 1), one of the main characteristic kavalactones, was isolated from kava–kava extract by chromatographic separation, and investigated in its ability to form a supramolecular complex with β-cyclodextrin (β-CyD). CyDs are cyclic oligosaccharides that can interact with a wide variety of drugs and the formation of their supramolecular complexes can influence the dissolution rate, the aqueous solubility and permeation ability of the drugs improving their bioavailability profile [3]. In particular, in this study cyclodextrins were selected for their properties as carriers by keeping the hydrophobic drug molecules in the polar media and deliver them to the surface of the biological membrane, e.g. skin. In the literature, we found only one report about the interaction between CyDs and kava–kava constituents [4]. However, no data concerning the formation and characterization of inclusion complexes were reported in this preliminary evaluation of the interaction between a kava–kava extract or an artificial mixture of kavalactones and β- and γ-CyD. In the present investigation β-CyD was selected for evaluating the possible formation of inclusion complexes with kavalactones because

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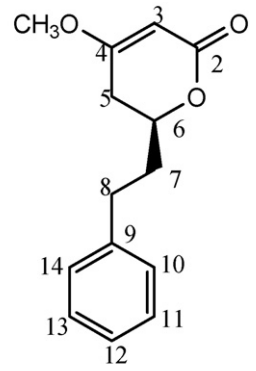

(*S*)-7,8-Dihydrokavain
(DHK)

Fig. 1. Chemical structure of (*S*)-7,8-dihydrokavain (DHK).

stationary phases based on β-cyclodextrin derivatives have been reported for chromatographic separations of kavalactones [5,6]. Moreover, β-CyD has been widely used in pharmaceutical applications because of its ready availability, low cost and cavity size suitable for the widest range of drugs [7]. Aim of this study was to provide a detailed structure and dynamic picture of the binding of the selected kavalactone DHK to β-CyD through circular dichroism (CD), NMR (^{1}H NMR complexation shifts analysis, ^{1}H NMR diffusion measurements, and ROESY) and by molecular dynamics simulations. The influence of β-CyD on the DHK solubility was also investigated by phase-solubility studies.

2. Materials and methods

2.1. Chemicals

Methanol was HPLC grade from Merck (Darmstadt, Germany). 85% Formic acid was purchased from Sigma–Aldrich (Fluka Chemicals, Sigma–Aldrich Division, Milano, Italy). Water was purified through a Milli-Q_{plus} system from Millipore (Milford, MA). Ethanol 96%, methanol and ethyl acetate were purchased from Riedel-de Haën Laborchemikalien GmbH & Co. KG. n-Hexane was purchased from Lab-Scan, Dublin. Dichloromethane and β-Cyclodextrin were purchased from Sigma–Aldrich. Spectroscopy-grade solvents (water and methanol) were purchased from Sigma–Aldrich. Deuterated solvents (D_2O, 99.8% and CD_3OD, 99.5%) were purchased from Merck.

2.2. Plant material

A commercial extract of kava–kava (lyophilized extract, lot no. 9A3847) was kindly offered by Aboca S.p.A. (Sansepolcro, Arezzo, Italy).

2.3. Isolation of (S)-7,8-dihydrokavain from the kava–kava extract

The lyophilized extract (3 g) was suspended in 4 mL of a solution of CH_2Cl_2/CH_3OH 1:1 and purified by column chromatography (Silica 9385, Kieselgel 60 Merck) at room temperature (elution mixture: hexane/ethyl acetate from 95:5 to 70:30, v/v). The fractions corresponding to DHK were collected, evaporated and crystallized from hexane, to obtain a white solid that was identified by HPLC/DAD/MS [8].

2.4. HPLC-DAD and HPLC-MS apparatus and methods

The HPLC system consisted of a HP 1100L instrument with a Diode Array Detector and managed by a HP 9000 workstation (Hewlett & Packard, Palo Alto, CA, USA). The reverse-phase column was a Prodigy ODS3 (5 μm, 150 × 2 mm, 100 Å, Phenomenex Torrance, CA, USA) maintained at 40 °C.

The eluents were: H_2O adjusted to pH 3.2 by HCOOH (A), and MeOH (B). The following solvent gradient was applied: 0–5 min A 70–50%; 5–10 min A 50–45%; 10–15 min A 45%; 15–30 min A 45–40%; 30–35 min A 40–30%; 35–40 min A 30–70%. The injected volume of sample was 10 μL of 1 mg/mL solution, the flow was 0.4 mL/min. UV–vis spectra were recorded in the range 200–590 nm, and chromatograms were acquired at 240, 254, 270, 350 and 590 nm.

The HPLC system was interfaced with a HP 1100 MSD API-electrospray (Hewlett & Packard, Palo Alto, CA, USA). The same column, mobile phase, time period and flow rate were used. Mass spectrometry operating conditions were optimised in order to achieve maximum sensitivity values: gas temperature 350 °C at a flow rate of 10 L/min, nebulizer pressure 30 p.s.i., quadrupole temperature 30 °C, and capillary voltage 3500 V. Full scan spectra from *m/z* 100 to 800 in the negative and positive ion mode were obtained (scan time 1 s).

2.5. Colyophilized products

Equimolar colyophilized products were prepared by freeze-drying (Lyovac GT2, Leybold-Heraeus) a solution obtained from a portion of the physical mixture of kavalactone/CyD dissolved in water and EtOH, followed by evaporation of the organic solvent. The 1:1 molar ratio of the dried powder was demonstrated through NMR.

2.6. Solubility studies

Phase-solubility studies were carried out in water, according to the method previously reported by Higuchi and Connors [9]. Briefly, excess amounts of DHK were added to water containing increasing concentrations of β-CyD (2–10 mM) and suspensions were shaken at constant temperature (25 ± 0.5 °C) for 1 day. After the equilibrium was reached, an aliquot was centrifuged and DHK concentration was determined by HPLC-DAD analysis. Each experiment was performed in triplicate (coefficient of variation (CV) <5%).

2.7. Spectroscopic measurements

NMR spectra were recorded at 298 K on a Varian INOVA 600 spectrometer operating at 14.1 T and referenced to the residual signal of HDO at 4.79 ppm. ROESY spectra were acquired using the standard pulse sequence with 0.6–0.8 s mixing times and continuous wave spin-lock (TROESY) with B_1 = 2800 Hz. DOSY experiments were performed with the DgcsteSL pulse sequence [10] using gradient pulses having 2 ms width and 1.17–70.5 G/cm strength. The samples consisted in the pure constituents and the colyophilized mixture kavalactone/β-CyD. The solids, in a total amount of about 3 mg, were dissolved in 0.5 mL 1:1 (v/v) CD_3OD/D_2O and sonicated for 10 min prior to perform measurements. The resulting solution was not completely clear even after filtration but this did not lead to apparent distortions of the NMR spectra.

CD spectra were recorded with a Jasco J-715 spectropolarimeter, using 0.02–0.2 cm cells, with the following conditions: bandwidth 1 nm, response 2 s, scanning speed 20 nm/min. The samples consisted in DHK 0.65 mM in CH_3OH/H_2O and a 1:1 colyophilized mixture of DHK/β-CyD 0.07 mM in H_2O.

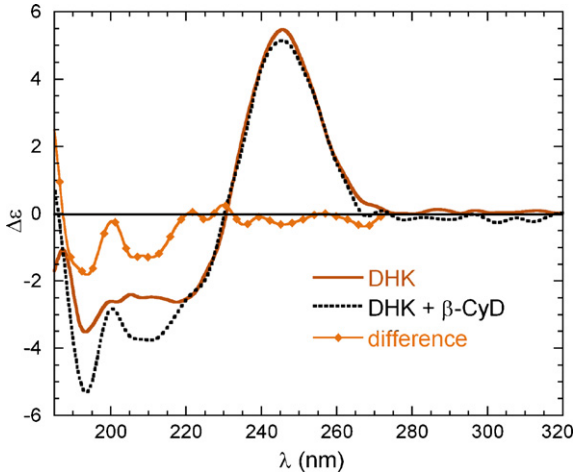

Fig. 2. CD spectrum in CH_3OH/H_2O of DHK free and bound to β-cyclodextrin, and their difference.

2.8. Computations

All calculations were run with Hyperchem 7.5 (Hypercube Inc., Gainesville, FL) using the Amber force field. Molecular dynamics simulations were run with the following parameters: heating time (from 0 to 300 K) 100 ps, simulation time 1500 ps at 300 K, step 1 fs, periodic box volume 20 Å × 20 Å × 20 Å, containing 320 water molecules. Starting geometries were generated by placing the guest with its long axis parallel to β-CyD axis outside the wide rim, then progressively translating inside by 0.5 Å steps, for 22 steps overall. All simulations leading to inclusion relaxed toward a structure with the phenyl ring inside the CyD cavity.

3. Results and discussion

3.1. Circular dichroism analysis

Equimolar coliophilized mixtures of DHK/β-CyD (10 mM) were dissolved in a 1:1 water/methanol solvent system. It is well known that the inclusion of organic chromophores into CyD cavities can induce CD bands allied to the electronic transitions of the chromophores (or alter the pre-existing CD signals if the guest is chiral) [11]. The CD spectrum of enantiopure (*S*)-DHK in CH_3OH/H_2O (Fig. 3) has a positive absorption at 245 nm and of a series negative signals in the 185–230 nm region, with minima at 217 and 193 nm; the spectrum of DHK sample is consistent with that reported for (6*S*) configuration [12,13]. The most red-shifted band is related to the $\pi-\pi^*$ transition of the α,β-unsaturated δ-lactone chromophore [14]. Further transitions are also displayed in the high energy region, where the $\pi-\pi^*$ transitions of the phenyl ring (^1L_a and ^1B_b) dominate the CD spectrum [15,16]. The CD spectrum of DHK/β-CyD coliophilized mixture (Fig. 2) is superimposable to that of DHK alone in the low energy region, while clear differences appear in the high energy region, showing a 50% intensity increase of the negative band. Thus, CD spectroscopy is consistent with the host/guest association, and also provides a first information concerning the geometry of the complex: the inclusion would mainly involve the phenyl ring [15,16], leaving the lactone moiety outside CyD.

3.2. NMR

NMR provides a detailed picture of the inclusion complexes, at least through three different and independent sets of information [17]. First, NMR can be used to measure complexation shifts, i.e. the difference between free and bound resonance frequency (in ppm) for the same nucleus. Second, through space proximity of nuclei of the host and the guest, can be quantitatively monitored by means of intermolecular Overhauser effects, measured through ROESY. Finally, the translational diffusion coefficient *D* can be measured through stimulated spin-echo experiments and variations of *D* on the can be related to the formation of host–guest complexes, as will be further discussed below [18].

Table 1
^1H chemical shifts (600 MHz) of dihydrokavain (in CD_3OD/D_2O) free and in mixture with β-cyclodextrin, and free-to-mixture differences (largest figures in bold).

Proton	δ (ppm) free	δ (ppm) mix	Δδ (ppm)
H3	5.15	5.10	−0.05
$H5_{ax}$	2.33	2.37	0.04
$H5_{eq}$	2.53	2.52	−0.01
H6	4.20	4.39	**0.19**
$H7_a$	1.86	1.89	0.03
$H7_b$	2.09	2.01	**−0.08**
$H8_a$	2.73	2.66	**−0.07**
$H8_b$	2.73	2.72	−0.01
o-Ph	7.11	7.21	**0.10**
m-Ph	7.23	7.26	0.03
p-Ph	7.19	7.16	−0.03
Me	3.71	3.67	−0.04

Table 1 reports the complexation shifts (Δδ) found for DHK. Most of the shifted protons are represented by the phenylethyl moiety of DHK, which is apparently responsible of the complexation. Surprisingly, also proton H6 of the lactone moiety showed a shift. The rationale of this effect was found only after the analysis of the complex through the molecular modelling experiments, as reported in Section 3.3.

The network of intermolecular Overhauser effects for DHK included in β-CyD is depicted in Fig. 3. The most relevant interactions are between the proton H3′ of CyD and the *ortho* positions on the phenyl ring of DHK, and between H5′ of CyD and the *meta*/*para*

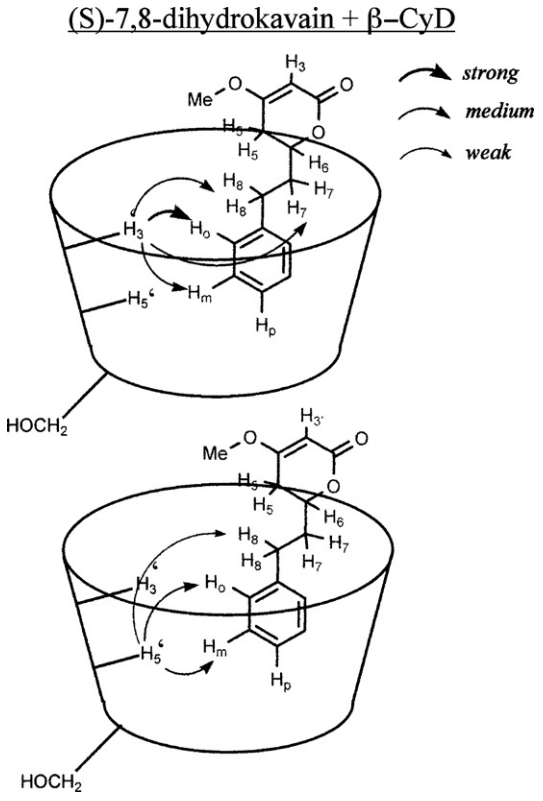

Fig. 3. Network of intermolecular nOe's between H-3′ and H-5′ of β-CyD and protons of DHK.

protons of the phenyl moiety of DHK. These data confirm a deep inclusion of DHK with the apolar aromatic tail in the hydrophobic pocket of β-CyD, oriented toward the primary hydroxyl rim. Consequently, the lactone group hangs out from the larger rim. The absence of other intermolecular nOes is a first strong indication of the existence of one well-defined 1:1 complex only. Different adduct stoichiometries (for example, β-CyD/guest 2:1) would necessarily lead to other interactions, e.g. involving the pyrone group and β-CyD or two different β-CyD molecules. This conclusion is also confirmed by the diffusion measurements discussed below.

While the above data provide a complete picture of the complex structure, they do not lend themselves to speculations on the magnitude of the association constant. This is due to the fact that the system is in fast exchange on the NMR timescale. Consequently resonances are found at the weighted average between the positions of the free and bound form and while the chemical shift of the former is known, for the latter it should need to be extrapolated. To this end, titration experiments are often used, but a precise determination of the thermodynamic constant was beyond our scope. To evaluate the mole fraction of DHK bound to β-CyD, we followed an alternative route, by means of diffusion measurements through PGSE technique. This experiment is nowadays very easily performed with NMR instruments equipped with *z*-gradient coils [10]. Firstly it is measured the diffusion coefficient of the guest alone D_F, secondly of the host alone D_H, and finally of the mixture D_{mix}, in solution [19]. Then, if the host is large enough, the diffusion coefficient of the bound complex can be approximated to that of the host alone ($D_B \approx D_H$), according to the facts that complexation represent only a minor perturbation to the host. In such a case, the following equation holds:

$$x_B = \frac{D_F - D_{mix}}{D_F - D_B} \approx \frac{D_F - D_{mix}}{D_F - D_H}$$

where x_B is the mole fraction of the bound drug.

The DOSY spectra in Fig. 4 clearly demonstrate that in the presence of β-CyD the translational diffusion of DHK is sizably slowed down with respect to the free drug, although it is not completely reduced to that of the macrocycle. This confirms that we are indeed in the presence of a free/bound equilibrium, and by means of the previous equation we can estimate the extent of inclusion to about $x_B \approx 80\%$ for (*S*)-7,8-dihydrokavain in 1:1 D_2O/CD_3OD.

3.3. Molecular dynamics simulations

Molecular mechanics-based calculations represent a powerful tool for investigating the geometry of cyclodextrin inclusion complexes [20]. In the current study, some insights into the inclusion

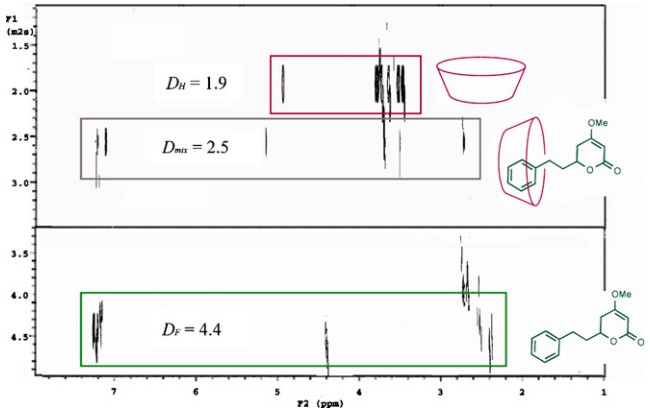

Fig. 4. DOSY spectra (600 MHz) in CD_3OD/D_2O of DHK (*F*, bottom) and of DHK/β-CyD mixture (top), displaying signals for the guest (*mix*) and the host (*H*); diffusion coefficients *D* shown in 10^{-10} m^2/s.

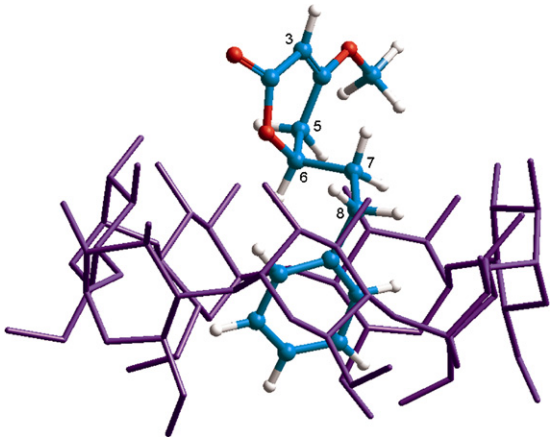

Fig. 5. Representative Amber structure of DHK/β-CyD inclusion complex obtained after MD simulations.

mode could be gained by molecular dynamics (MD) simulations using the Amber force field. The binary system DHK/β-CyD was immersed in a bath of water molecules; a set of different starting geometries was obtained by progressively inserting the DHK within the β-CyD cavity, with the long axis parallel to the β-CyD ring axis. MD simulations were then run at 300 K; those leading to inclusion systematically relaxed toward structures (one example showed in Fig. 5) with the phenyl ring included in the β-CyD cavity, with its C1′–C4′ direction slightly tilted (15–20°) with respect to the β-CyD ring axis.

Interestingly enough, an inversion of the pyrone ring is observed between free and bound DHK: thereby the phenylethyl substituent at C6 switches between the equatorial position in the free state, and the axial in the complex (Fig. 5).

Conversely, the hydrogen H6 should be axial in the free and equatorial in the bound form, and we can predict that upon binding, proton H6 should experience a downfield shift, which matches the experiment, where we observe a positive $\Delta\delta = 0.1$, as reported in Table 1. Thus, MD results corroborate and further clarify observed spectroscopic data.

3.4. Phase-solubility study

(*S*)-7,8-Dihydrokavain (DHK) is practically insoluble in water, while β-cyclodextrin is moderately soluble (about 18 mg/mL at 25 °C). The phase-solubility diagram of (*S*)-7,8-dihydrokavain with β-CyD (Fig. 6) displayed a B_s curve [9]. Solubility was increased twice in the range of 2–4 mM of β-CyD.

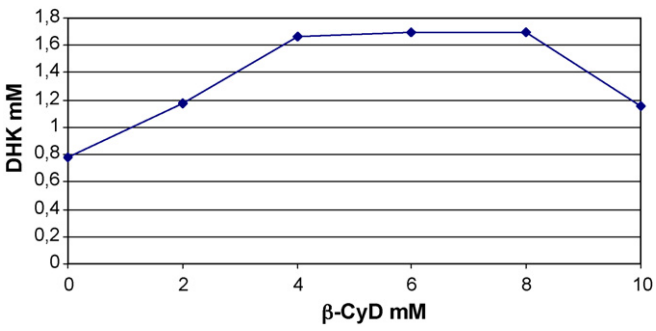

Fig. 6. Phase-solubility diagram of DHK/β-CyD system.

4. Conclusions

In this paper NMR and UV-CD experiments are extensively used for the complete characterization of the complex β-CyD/DHK. In particular DOSY experiments demonstrated that in the presence of β-CyD the translational diffusion of kavalactone is sizably slowed down (2.5×10^{-10} m^2/s) with respect to the free DHK (4.4×10^{-10} m^2/s) according to the inclusion of DHK in the cavity of (β-CyD). ROESY experiments confirmed the inclusion of DHK in the hydrophobic pocket of β-CyD through the primary hydroxyl rim, being the most relevant interactions between the H3′ of β-CyD and the *ortho* protons on the phenyl ring of the kavalactone, and between H5′ of β-CyD and the *meta*/*para* protons of DHK phenyl ring. The inclusion of DHK through the phenyl ring, leaving the lactone moiety outside of the larger rim of CyD was also confirmed by the induced CD effects. The binary solution DHK/β-CyD presents a 50% intensity increase of the negative band of the π–π^* transitions of the phenyl ring. CD and NMR, especially the innovative DOSY experiments, were found powerful methods for the complete and unambiguous characterization of the inclusion complex. Moreover, molecular dynamics simulations results corroborated and further clarify observed spectroscopic data. It was observed that the phenylethyl moiety at C6 occupies a preferential equatorial position in the free state, and an axial one in the complex, justifying the large measured downfield shift experienced by H6 of DHK upon binding. Finally the influence of β-CyD on water solubility of DHK was demonstrated by phase solubility studies. In the range 2–4 mM of β-CyD, solubility of DHK was increased only two-fold, but being β-CyD also a penetration enhancer, *in vivo* studies will be performed to clarify a possible role of the complex on the bioavailability of DHK. At the same time, the studies will be extended to other CyDs in order to find hosts can increase deeply water solubility of DHK.

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