

Investigation of inclusion complex of Epothilone A with cyclodextrins



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ABSTRACT

The inclusion complexation of Epothilone A with native cyclodextrin (β - or γ -CD) and its derivative hydroxypropyl- β -cyclodextrin (HP β CD) were prepared. Their behavior, characterization, and binding ability were investigated in both solution and the solid state by means of UV–vis, NMR, XRD, DSC and SEM. The results show that the water solubility and solution stability obviously increased in the inclusion complex with cyclodextrins. Meanwhile, the inclusion complexes still retained anticancer activity against A549 and MCF-7 cells, similar to free Epothilone A. This satisfactory water solubility, high solution stability, and high anticancer activity of the Epothilone A/CD complexes will be potentially useful as an anticancer therapy.

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1. Introduction

Epothilone A and its analogs are naturally occurring microtubule-stabilizing macrolides that were first isolated from the myxobacterium *Sorangium cellulosum* by Reichenbach, Höfle, et al. in 1987 (Bollag et al., 1995; Höfle et al., 1996). Epothilones are active against human cancer cell proliferation (Gerth, Bedorf, Hofle, Irschik, & Reichenbach, 1996) and have served as successful lead structures in the development of new anticancer drugs (Altmann, Pfeiffer, Arseniyadis, Pratt, & Nicolaou, 2007; Rivkin, Chou, & Danishefsky, 2005). At least seven epothilone-derived agents (including the natural product Epothilone B) have entered human clinical evaluation. The most advanced of these analogs, the Epothilone B lactam BMS-247550 (ixabepilone), has recently obtained FDA approval for clinical use among cancer patients (Lechleider et al., 2008).

Epothilones have a taxane-like mechanism of action, but in contrast to paclitaxel (PTX) or docetaxel, they inhibit the growth of human cancer cells *in vitro* by inducing overexpression of the P-glycoprotein efflux pump (Lee et al., 2009). This indicates that they might be more efficient in the treatment of multidrug-resistant tumors (Altmann et al., 2007). Epothilone A is 16-membered

macrolide in comparison with taxanes, and binds to a similar (but not identical) pharmacophore on the β -tubulin subunit of microtubules (Lee et al., 2009). Experimental data and three-dimensional quantitative structure-activity relationship studies (3D QSAR) have suggested that epothilones, especially Epothilone B, are more potent inducers of tubulin dimerization than taxanes, because they bind to tubulin with higher affinity than taxanes do (Forli, Manetti, Altmann, & Botta, 2010). However, poor aqueous solubility and stabilization restrain the usage of Epothilone A as an anticancer drug. It is necessary to search for an efficient and non-toxic carrier for Epothilone A to extend its therapeutic applications. The most commonly used method to improve the physicochemical properties of drug molecules is the preparation of inclusion complexes with cyclodextrins (CDs) (Davis & Brewster, 2004; Loftsson & Duchene, 2007; Loftsson, Hreinsdottir, & Masson, 2005).

It is well-known that cyclodextrins (CDs) are non-toxic macrocyclic oligosaccharides consisting of (α -1,4)-linked α -L-glucopyranose units with a hollow hydrophobic interior and a hydrophilic outer surface. These CDs can encapsulate various inorganic or organic molecules, which enter partly or entirely into the relatively hydrophobic cavity of CDs and simultaneously expel a few high-energy water molecules from the interior (Karathanos, Mourtzinos, Yannakopoulou, & Andrikopoulos, 2007). This usually enhances drug solubility in aqueous solutions and affects the chemical characteristics of the encapsulated drug, which are advantageous in the pharmaceutical industry (Liu & Chen, 2006; Misiuk & Zalewska, 2009; Wu, Liang, Yuan, Wang, & Yan, 2010). However, to the best of our knowledge, no scientific study on the inclusion

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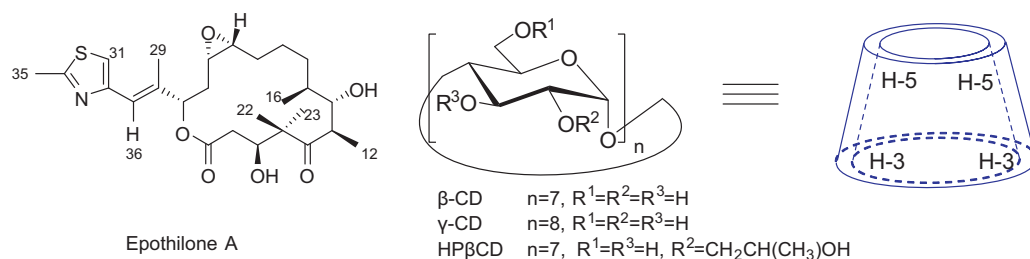


Fig. 1. The structures of Epothilone A, β-CD, γ-CD, and HPβCD.

behavior of Epothilone A/CD complexes has hitherto been reported. More recently, our group has reported that the inclusion complexation of CDs with natural products such as GA-13315 (Yang et al., 2012), taxifolin (Yang et al., 2011), crassicauline A (Chen et al., 2011), nimbin (Yang et al., 2010), artemether (Yang, Lin, Chen, & Liu, 2009), scutellarin (Yang, Yang, Lin, Chen, & Liu, 2009), and azadirachtin B (Yang, Chen, Lin, & Liu, 2008) significantly enhanced the water solubility and bioavailability of the products. Thus, taking into account this ability of cyclodextrins, we have investigated the inclusion complexes of Epothilone A with cyclodextrins.

In this paper, we aim to report the preparation and characterization of some water-soluble inclusion complexes formed by Epothilone A and native cyclodextrin (β- or γ-CD) and its derivative: 2-hydroxypropyl-β-cyclodextrin (HPβCD) (Fig. 1). We were particularly interested in exploring the solubilization effect of CDs on Epothilone A and the binding ability of the resulting inclusion complexes, which would potentially provide a useful approach for obtaining novel Epothilone-based healthcare products with high water solubility, high bioavailability and low toxicity.

2. Materials and methods

2.1. Materials

Epothilone A (FW = 493.66, purity > 99%) was obtained from Sigma-Aldrich (Dorset, UK). β-CD (average substitution degree = 1135), γ-CD (average substitution degree = 1297), and 2-hydroxypropyl-β-cyclodextrin (HPβCD, average substitution degree = 1380) were purchased from ABCR GmbH and Co. KG and used without further purification. Other reagents and chemicals were of analytical reagent grade. All experiments were carried out using ultrapure water.

2.2. Apparatus

NMR spectra were conducted on a Bruker Avance DRX spectrometer at 500 MHz and 25 °C in D₂O. The one-dimensional spectra of both solutions were run with an FID resolution of 0.18 Hz/point. The residual HDO line had a line width at a half-height of 2.59 Hz. Two-dimensional (2D) ROESY spectra were acquired at 25 °C with presaturation of the residual water resonance and a mixing (spin-lock) time of 350 ms at a field of ~2 kHz, using the TPPI method, with a 1024 K time domain in F2 (FID resolution 5.87 Hz) and 460 experiments in F1. Processing was carried out with zero-filling to 2 K in both dimensions using sine (F2) and qsine (F1) window functions, respectively.

The UV-vis spectrum was recorded on a Shimadzu UV 2401 spectrophotometer (Japan) equipped with a conventional 1 cm path (1 cm × 1 cm × 4 cm) quartz cell in a thermostated compartment, which was kept at 25 °C by a Shimadzu TB-85 Thermo Bath unit.

A powder X-ray diffraction spectrum was taken by a Rigaku TTRIII Rotating Target diffractometer with Cu Kα radiation (40 kV,

100 mA), at a scanning rate of 5 °/min. Powder samples were mounted on a vitreous sample holder and scanned with a step size of $2\theta = 0.02^\circ$ between $2\theta = 3^\circ$ and 50° .

Differential scanning calorimetry (DSC) measurements were conducted on a 2960 SDT V3.0F instrument, and 3–3.5 mg of each sample was heated at a rate of 10 °C/min from room temperature to 400 °C under a dynamic nitrogen atmosphere at a flow rate of 70 mL/min.

SEM photographs were determined on an FEI QUANTA 200. The powders were previously fixed on a brass stub using double-sided adhesive tape and then were made electrically conductive by coating with a thin layer of gold (approximately 300 Å) in a vacuum for 30 s and at 30 W. The pictures were taken at an excitation voltage of 15, 20 or 30 kV and a magnification of 1080, 1200, 1400 or 2000×.

2.3. Preparation of Epothilone A/β-CD, Epothilone A/γ-CD and Epothilone A/HPβCD complexes

Epothilone A (0.03 mM, 15 mg) and CD (0.01 mM) were completely dissolved in a mixed solution of ethanol and water (ca. 20 mL, v:v = 1:4; given the poor water solubility of Epothilone A, ethanol was used), and the mixture was stirred for 3 days at room temperature. After evaporating the ethanol from the reaction mixture, the uncomplexed Epothilone A was removed by filtration. The filtrate was evaporated under reduced pressure to remove the solvent and dried in a vacuum to produce the Epothilone A/CDs complexes. Epothilone A/β-CD complex (yield = 80): ¹H NMR (500 MHz, D₂O, TMS): δ 7.08 (s, 1H, H-31' of the thiazole ring proton for Epothilone A), 6.58 (s, 1H, H-28' of the double bond proton for Epothilone A), 5.04 (s, 7H, H-1 of β-CD), 3.45–3.95 (m, 42H, H2–H6 of β-CD), 1.0–3.3 (m, proton for Epothilone A). Epothilone A/γ-CD complex (yield = 80): ¹H NMR (500 MHz, D₂O, TMS): δ 7.17 (s, 1H, H-31' of the thiazole ring proton for Epothilone A), 6.50 (s, 1H, H-28' of the double bond proton for Epothilone A), 5.01 (s, 7H, H-1 of γ-CD), 3.40–3.85 (m, 56H, H-2–6 of γ-CD), 1.0–3.3 (m, proton for Epothilone A). Epothilone A/HPβCD-CD complex (yield = 80): ¹H NMR (500 MHz, D₂O, TMS): δ 7.06 (s, 1H, H-31' of the thiazole ring proton for Epothilone A), 6.48 (s, 1H, H-28' of the double bond proton for Epothilone A), 4.99–5.16 (s, 7H, H-1 of HPβCD), 3.30–4.15 (m, 100H, H-2–6 and CH₂- and CH₃-2, 3, 6 of HPβCD).

2.4. Preparation of Epothilone A/β-CD, Epothilone A/γ-CD and Epothilone A/HPβCD physical mixture

The physical mixture, to test for possible inclusion, was prepared by mixing the powders in a 1:1 molar ratio of Epothilone A and CDs in an agate mortar.

2.5. Standard curve of Epothilone A

A series of Epothilone A ethanol solutions with concentrations ranging from 8.0×10^{-6} M to 7.3×10^{-5} M were prepared. The

measurements were performed using a UV–vis spectrophotometer (Shimadzu UV 2401, Japan) at 248 nm. A standard curve was then prepared using the concentration (C , mM) as the x -coordinate and the absorbance (A) as the y -coordinate. The standard curve of Epothilone A can be expressed by $A = 11501.96C + 9.3 \times 10^{-4}$ ($R^2 = 0.999$).

2.6. Phase solubility test

Phase solubility studies were performed according to the methods described by Higuchi and Connors (1965). An excess amount of Epothilone A was suspended in ultrapure water containing increasing amounts of β -CD, γ -CD, and HP β CD (from 0 mM to 10.00 mM). The mixtures were placed in an ultrasonic bath for 2 h at 25 °C in the dark and were then left in the dark for 24 h. After Epothilone A was prepared, the mixtures were withdrawn and subsequently filtered through a 0.45 μ m hydrophilic membrane filter. All samples were prepared in triplicate. The concentration of Epothilone A in the filtrate was determined at 248 nm using a Shimadzu UV 2401 spectrophotometer (Japan). The phase solubility profiles were obtained by plotting the solubility of Epothilone A vs. the concentration of β -CD, γ -CD or HP β CD. The apparent stability constant, K_c of the Epothilone A and β -CD, γ -CD or HP β CD complexes can be calculated from the slope and the intercept of the linear segment of the phase solubility line using the following equation:

$$K_c = \frac{k}{S_0(1-k)} \quad (1)$$

where S_0 is the intrinsic solubility of Epothilone A in ultrapure water in the absence of β -CD, γ -CD or HP β CD and k is the slope of the straight line ($S_0 = 0.1254$ mg/mL) (Dimarco et al., 2002).

2.7. Stoichiometry determination: Job's method

The continuous variation method was performed in order to confirm the stoichiometry of the complex. The sum of the concentration of both components was kept constant ($[\text{Epothilone A}] + [\text{CD}] = 10 \times 10^{-4}$ M) whilst the molar fraction of Epothilone A ($R = [\text{Epothilone A}] / ([\text{Epothilone A}] + [\text{CD}])$) was varied from 0.0 to 1.0. After stirring for 48 h, the UV absorption at 248 nm was measured for all solutions and the difference in the absorption between that in the presence (A) and absence of CD (A_0), $\Delta A = A - A_0$, was plotted versus the molar fraction R . The maximum amount of the complex should occur at the stoichiometric ratio.

2.8. Aqueous solubility of Epothilone A/CDs

An adequate amount of the complex was added to 2 mL of water (ca. pH 7.0) to ensure that the solution reached saturation under nitrogen, sheltered from light, and the mixture was stirred for 1 h at 20 ± 2 °C. Then, the remaining solid in the solution was filtered off using a 0.45 μ m cellulose acetate membrane. The filtrate was evaporated under reduced pressure to dryness and the residue was dosed by the weighing method.

2.9. Measurement of cytotoxicity

The cytotoxicity of the samples on tumor cells was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Chen et al., 2008). Human lung adenocarcinoma cells (A549) were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, and human breast cancer cells (MCF-7) were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum. Firstly, cells were grown in 96 well plates at 6000 cells per well in a final volume of 200 μ L culture medium (A549: 10% of RPMI-1640, MCF-7: 10% of DMEM)

per well. Then, the cells were cultured in an incubator (5% CO₂, 37 °C) until the cells reached 70–80% confluency. Experimental drugs were added at the indicated concentration of 0.06 μ mol/mL. After culture, the cells were kept in the incubator for 24 h, at which point 10 μ L of MTT were added to each well and incubated for 4 h. The culture medium was discarded and 100 μ L of DMSO were added to each well, then swirled gently in the dark for 15 min. The absorbance was measured in each well at 570 nm using a microtiter plate reader, and the average value was determined from triplicate readings. The inhibition of cell proliferation was calculated by the following formula:

$$\text{growth inhibition (\%)} = \left(\frac{\text{OD}_{\text{control}} - \text{OD}_{\text{treated}}}{\text{OD}_{\text{control}}} \right) \times 100\%$$

2.10. Stability test

Comparative tests involving the stability of an aqueous solution of free and complexed Epothilone A with β -CD, γ -CD, and HP β CD were traced by absorbance changes in the UV spectra at room temperature (for 1500 min; all experiments were carried out in triplicate at predetermined time intervals). The results are expressed as the percentage of remaining Epothilone A, i.e., the A/A_0 100 ratio, where A_0 is the initial concentration of Epothilone A alone or the Epothilone A/CD complex and A is the concentration at the specified time point.

3. Results and discussion

3.1. Phase solubility diagrams

According to the phase solubility diagram results, the solubility of Epothilone A increased as a linear function of the CD concentration. The regression equations are as follows:

$$[\text{Epothilone A}] = 0.129[\beta\text{-CD}] + 1.753, \quad R^2 = 0.997$$

$$[\text{Epothilone A}] = 0.087[\gamma\text{-CD}] + 1.902, \quad R^2 = 0.988$$

$$[\text{Epothilone A}] = 0.073[\text{HP}\beta\text{CD}] + 2.173, \quad R^2 = 0.991$$

According to Higuchi and Connors's theory (Higuchi & Connors, 1965), these three relationships are classified as a typical A_L-type, suggesting that the stoichiometry of Epothilone A in the β -CD, γ -CD or HP β CD inclusion complex was 1:1. The apparent stability constant (K_c) value is most often between 50 M^{−1} and 2000 M^{−1} (Loftsson et al., 2005). In our study, the calculated K_c values of the Epothilone A/ β -CD, Epothilone A/ γ -CD and Epothilone A/HP β CD complexes were 583 M^{−1}, 375 M^{−1} and 310 M^{−1}, respectively, suggesting a moderate tendency of the drug to enter the CD molecule. The solubility of CD included Epothilone A is 22–24 times higher than that in water. Moreover, the complexation constants had the following order: $K_c(\beta\text{-CD}) > K_c(\gamma\text{-CD}) \approx K_c(\text{HP}\beta\text{CD})$. The decreased stability of the Epothilone A/HP β CD complex may be due to the steric hindrance of the propyl substituents, which prevented Epothilone A inclusion. The low stability of Epothilone A/ γ -CD complex may be due to fact that the binding free energy of β -CD is relatively larger than that of γ -CD, leading β -cyclodextrin complex is more stable (see supporting information).

The stoichiometry of complex formation between Epothilone A and CD was also determined using continuous variation Job's method. The Job's plot is shown in Fig. 4(b). As shown in the Job's plot, the maximum peak was obtained at $R = 0.5$, which indicates the formation of 1:1 inclusion complexes between Epothilone A and β -CD, γ -CD or HP β CD, in agreement with the phase solubility study (Job's plot of β -CD and HP β CD, see Supplementary data).

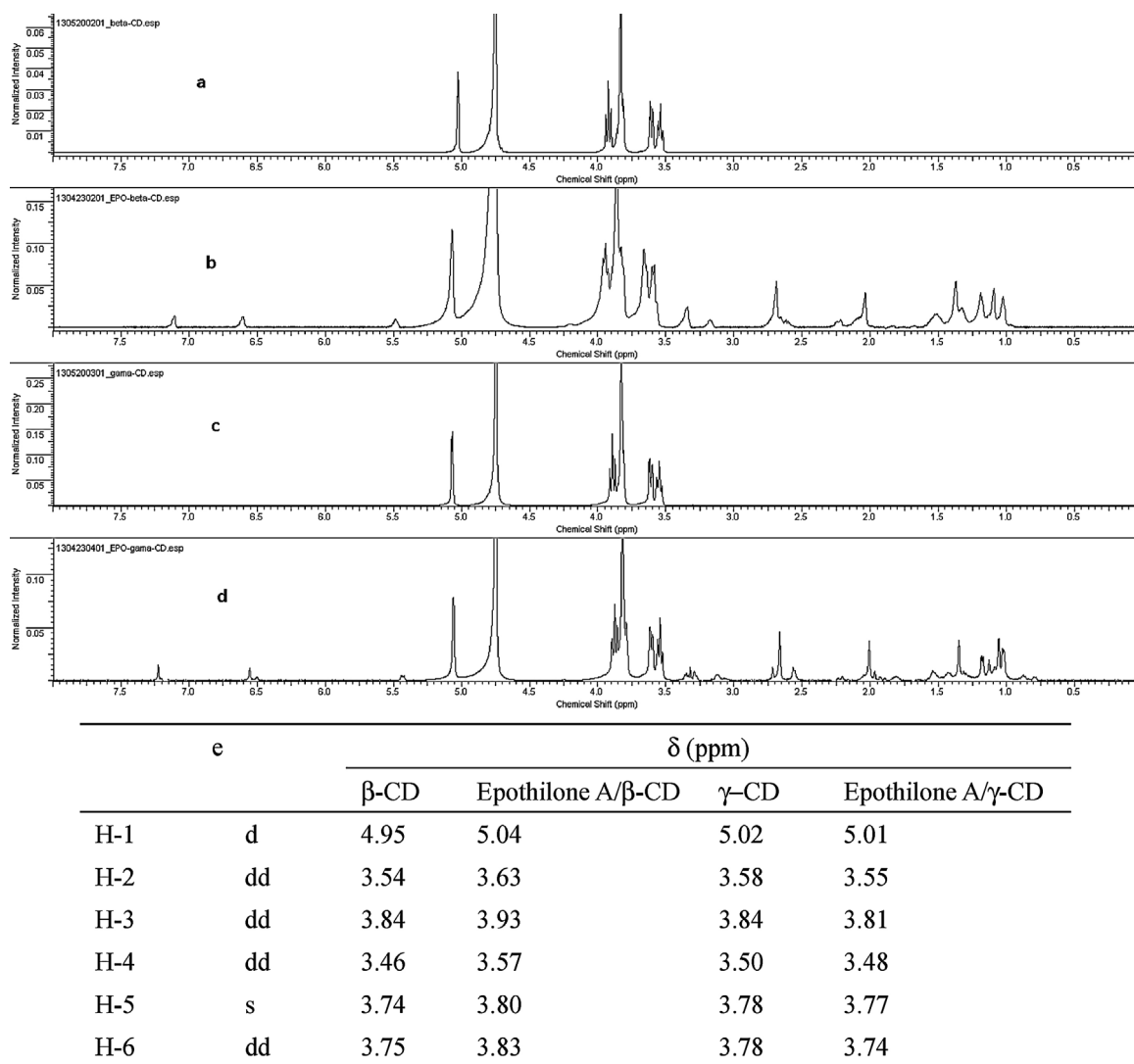


Fig. 2. ¹H NMR spectra of Epothilone A in the absence and presence of β-CD and γ-CD in D₂O at 25 °C, respectively. (a) β-CD, (b) Epothilone A/β-CD complex, (c) γ-CD, (d) Epothilone A/γ-CD complex; and (e) the chemical shifts (δ) of the β-CD, γ-CD, Epothilone A/β-CD and Epothilone A/γ-CD complex.

3.2. Characterization results and discussion

3.2.1. ¹H and 2D NMR analysis

NMR spectroscopy provides some of the most powerful evidence for the study of host–guest chemistry in solution, and the information provided by the chemistry shifts has been used to establish inclusion modes (Jiang et al., 2007). We compared the ¹H NMR spectra of Epothilone A in the presence of the host CDs (Fig. 2). Owing to its poor water solubility, Epothilone A is transparent to ¹H NMR under most conditions when D₂O is used as the solvent. Assessment of the Epothilone complex by ¹H NMR clearly demonstrated the presence of the framework protons of the Epothilone A molecule, consistent with significant solubilization. As illustrated in Fig. 2(a), the majority of Epothilone A protons displayed chemical shifts at δ 1.0–3.0 and 5.3–7.5 ppm, which were distinct from the CD protons (usually at δ 3.0–5.0). By comparing the integration area of these protons with that of the H-1 protons of the CDs, we calculated the inclusion stoichiometry of the Epothilone A/CD complexes as 1:1 for the Epothilone A/β-CD, Epothilone A/γ-CD and Epothilone A/HPβCD complexes.

Given that the β-CD H3 and H5 protons inside the hydrophobic cavity are close to the wide and narrow rims of the β-CD cavity, respectively, then significant chemical shifts at the H3 and H5 protons would result if the guest molecule located in the β-CD cavity

were to form an inclusion complex. Inclusion complexation with Epothilone A had a minor effect on the δ values of the H-5 and H-6 protons of β-CD (≤0.02 ppm). In contrast, the values of the H-1, H-2, H-3 and H-4 protons exhibited relatively weak but significant changes (0.03–0.06 ppm), which could have been caused by the hydrogen bond between the hydroxyl arms of β-CD and the nitrogen, sulfur and oxygen atoms of Epothilone A. It is worth noting that the H-3 protons shifted ca. 9 ppm, but the H-5 protons shifted only ca. 6 ppm after inclusion complexation. This phenomenon may indicate that Epothilone A may have inserted into the β-CD cavity from the narrower rim (Fig. 5A(a)). It was also revealed that Epothilone A should insert into the γ-CD and HPβCD cavities from the wide side (Fig. 5A(b)) (for HPβCD, see Supplementary data).

ROESY experiments are generally performed to determine the geometry of the inclusion complexes of organic molecules with CDs (Kemalbekov et al., 2011). Two protons that are closely located in space can produce a nuclear Overhauser effect cross-correlation in NOESY or ROESY. To gain more conformational information, we obtained 2D ROESY of the inclusion complexes of Epothilone A with CDs. The ROESY spectrum of the Epothilone A/β-CD complex (Fig. 3(a)) shows appreciable correlation of the H-29', H-31', H-35' and H-36' protons of Epothilone A with the H-3, H-5 and H-6 protons of β-CD (peak a). These results indicate that the thiazole ring of Epothilone A was included in the β-CD cavity. It was also shows

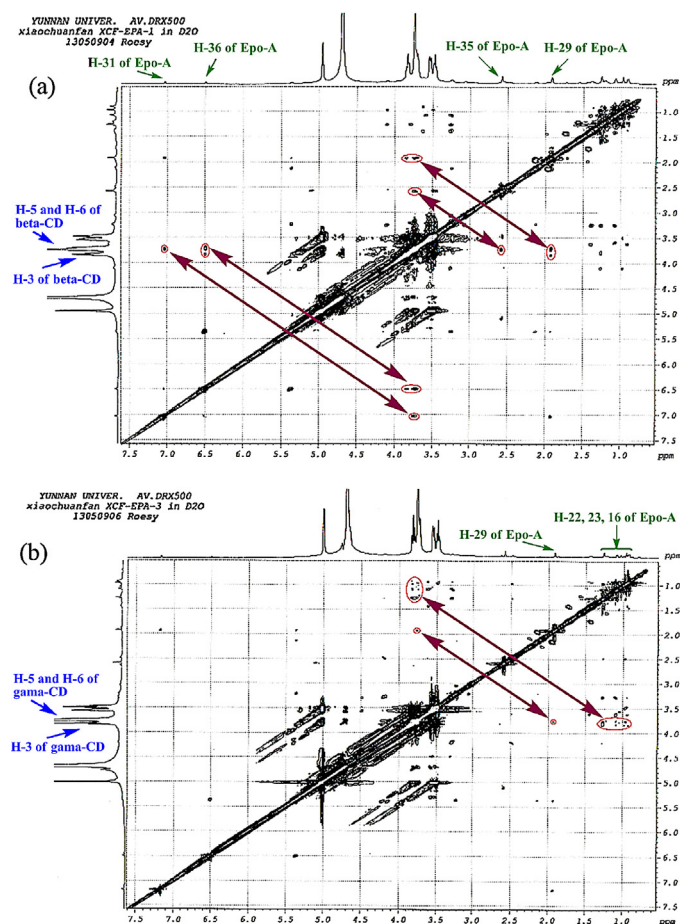


Fig. 3. ROESY spectrum of (a) Epithilone A/β-CD complex and (b) Epithilone A/γ-CD complex in D₂O.

that Epithilone A should be included in the HPβCD cavities in similar ways (see Supplementary data). The ROESY spectrum of the Epithilone A/γ-CD complex (Fig. 3(b)) showed significant correlations between the H-29', H-17', H-16', H-12', H-23' and H-22' protons of Epithilone A and the H-3, H-5 and H-6 protons of γ-CD (peaks b). These results indicate that the large lactone ring was included in the γ-CD cavity (Fig. 4).

Based on these observations, together with the 1:1 stoichiometry, we deduced the possible inclusion modes of Epithilone A with CDs, as illustrated in Fig. 5A.

3.2.2. FT-IR analysis

The FT-IR spectra of the pure Epithilone A, γ-CD, physical mixture and inclusion complex are illustrated in Fig. 5B. The FT-IR spectrum of γ-CD showed the prominent absorption bands at 3410 cm⁻¹ (for O–H stretching vibration), 2927 cm⁻¹ (for C–H stretching vibration), 1632 cm⁻¹ (for H–O–H bending), 1153 cm⁻¹ (for C–O stretching vibration), and 1028 cm⁻¹ (C–O–C stretching vibration) (Fig. 5B(b)). The FT-IR spectrum of Epithilone A consisted of prominent absorption bands with stretching vibrations of O–H (3497 cm⁻¹, 3435 cm⁻¹), C–H stretching (2959 cm⁻¹, 2877 cm⁻¹), stretching vibrations of C=O (1739 cm⁻¹), C=N stretching (1685 cm⁻¹) and C=C (1638 cm⁻¹), bending vibrations of –CH₂– (1457 cm⁻¹), –CH₃ (1382 cm⁻¹), and stretching vibrations of C–O (1155 cm⁻¹), C–N (1015 cm⁻¹), C–C (979.3 cm⁻¹) (Fig. 5B(a)). The spectrum of the physical mixture was equivalent to the simple combination of Epithilone A and γ-CD. Some characteristic absorption peaks of Epithilone A at 3497, 2959, 2877, 1739, 1685, 1155 cm⁻¹ were easy to observe (Fig. 5B(d)), suggesting

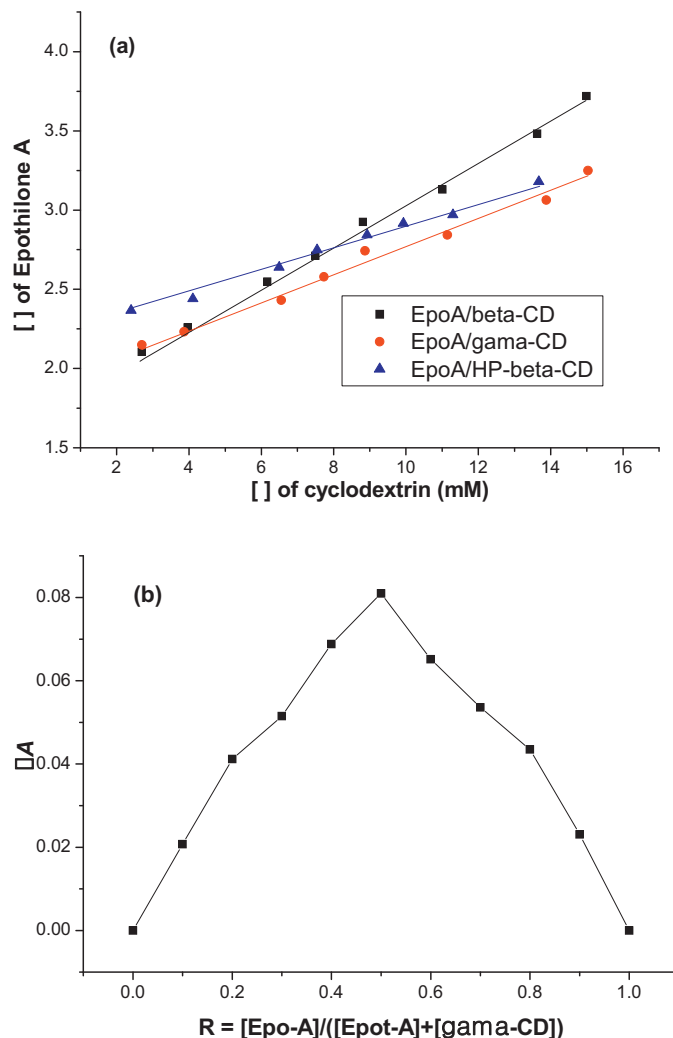
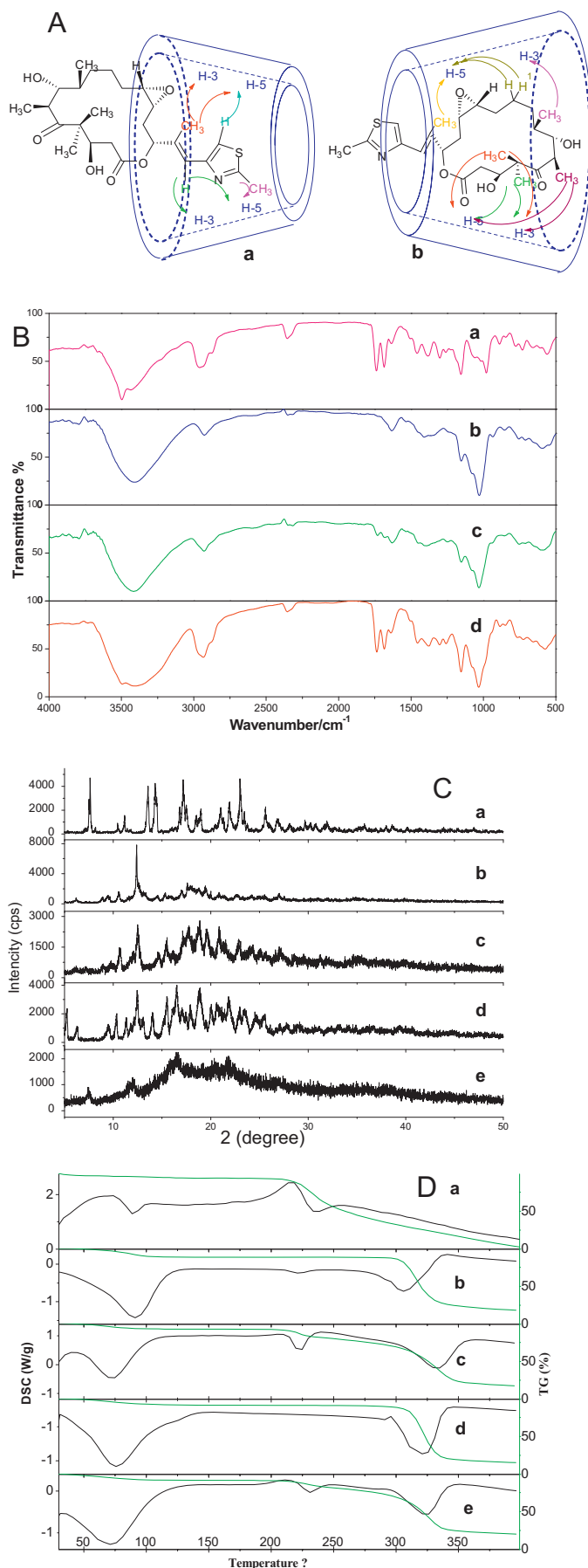


Fig. 4. (a) Phase solubility diagram of Epithilone A in water depending on the CD concentration (β-CD, γ-CD and HPβCD). (b) Job's continuous variation plot of the Epithilone A/γ-CD complex.

the natural structure of Epithilone A still existed without any interactions with γ-CD. For the inclusion complex, (Fig. 5B(c)) the spectrum was very similar to that of γ-CD and the characteristic peaks of Epithilone A almost entirely disappeared. In addition, no additional peak was detected in the spectrum of the inclusion complex, indicating the absence of any chemical reactions between Epithilone A and γ-CD. These results indicate that A Epithilone A/γ-CD inclusion complex was obtained and that the large lactone ring of Epithilone A entered the cavity of γ-CD during the formation of the inclusion complex. It was also revealed by the FT-IR spectra that Epithilone A should insert into the β-CD and HPβCD cavities (see Supplementary data).

3.2.3. XRD analysis

Powder X-ray diffractometry (XRD) is a useful method for the detection of CD complexation in the powder or microcrystalline states. The XRD patterns of Epithilone A, β-CD, HPβCD and γ-CD as well as their inclusion complexes are illustrated in Fig. 5C and D (the XRD of HPβCD and its inclusion complex with Epithilone A can be seen in the Supplementary data). As indicated in Fig. 5C, Epithilone A (Fig. 5C(a)), β-CD (Fig. 5C(b)) and γ-CD (Fig. 5C(d)) were in a crystalline form. In contrast, the XDR of the Epithilone A/β-CD and Epithilone A/γ-CD complexes (Fig. 5C(c) and Fig. 5C(e), respectively) are amorphous and show halo patterns, which are



quite different from the superimposition of crystalline Epothilone in β -CD and γ -CD, indicating the formation of an inclusion complex between β -CD (or γ -CD) and Epothilone A. A similar phenomenon was found for HP β CD and its inclusion complex (see Supplementary data).

In addition, most of the crystalline diffraction peaks of β -CD, γ -CD, and HP β CD disappeared after complexation with Epothilone A, indicating the interaction between β -CD (γ -CD or HP β CD) and Epothilone A.

3.2.4. Thermogravimetry analysis

The thermal properties of the Epothilone A/ β -CD, Epothilone A/ γ -CD, and Epothilone A/HP β CD complexes were investigated by thermogravimetric (TG) methods (see Supplementary data). A systematic analysis of the TG curves showed that Epothilone A decomposes at ca. 212°C, β -CD at ca. 303°C, γ -CD at ca. 310°C, and HP β CD at ca. 320°C (Fig. 5D). However, the thermal stability of their inclusion complexes differed; that is, the decomposition temperatures were ca. 215, 210, and 217°C for the Epothilone/ β -CD, Epothilone/ γ -CD and Epothilone/HP β CD inclusion complexes, while the Epothilone A/ β -CD and Epothilone A/HP β CD complexes possessed slight higher decomposition temperatures than Epothilone/ γ -CD (TG of HP β CD and Epothilone/HP β CD, see in the Supplementary data).

The differential scanning calorimetry (DSC) thermogram gave further information about the thermal properties of the Epothilone A/ β -CD, Epothilone A/ γ -CD, and Epothilone A/HP β CD complexes. As shown in Fig. 5D, the DSC curve of Epothilone A contained a sharp endothermic peak at 90.3°C, which is the melting temperature of Epothilone A. The thermal properties of Epothilone A and cyclodextrin complexes were further studied by the DSC method. As shown in Fig. 5D(a), the DSC curve of Epothilone A contained an another endothermic peak at 235°C. In contrast, the DSC curves of pristine β -CD, γ -CD, and HP β CD had an endothermic peak at 307, 322, and 358°C (Fig. 5D), respectively. However, in the DSC curves of the Epothilone A/CDs complexes, the endothermic peaks at about 235°C corresponding to free Epothilone A disappeared, coinciding with the appearance of a new endothermic peak at 222, 231, and 220°C in the case of the Epothilone A/ β -CD, Epothilone A/ γ -CD, and Epothilone A/HP β CD (Fig. 5D) complexes, respectively. These results further confirm the formation of Epothilone A/CDs complexes (DSC of HP β CD and Epothilone/HP β CD, see in the Supplementary data).

3.2.5. SEM analysis

Scanning electron microscopy (SEM) is a qualitative method used to study the structural aspects of raw materials, i.e., CDs and drugs or the products obtained by different methods of preparation, such as physical mixing, solution complexation, coevaporation, and others (de Araujo et al., 2008; Duchêne, 1987). The SEM photomicrographs of HP β CD, Epothilone A, their inclusion complexes and their physical mixtures are shown in Fig. 6. Pure Epothilone A existed as irregularly shaped crystals (Fig. 6(a)), and HP β CD crystallized as a spherical crystal with cavity structures (Fig. 6(b)). The physical mixture of Epothilone A/HP β CD revealed some similarities

Fig. 5. (A) Possible inclusion mode and significant ROESY ($\leftrightarrow$) correlations of the Epothilone A/ β -CD (a) and Epothilone A/ γ -CD complex (b). (B) Fourier transform infrared (FT-IR) spectra of (a) Epothilone A, (b) γ -CD, (c) Epothilone A and γ -CD complex at a 1:1 molar ratio, and (d) Epothilone A and γ -CD physical mixture. (C) XRD patterns: (a) Epothilone A, (b) β -CD, (c) Epothilone A/ β -CD inclusion complex, (d) γ -CD, and (e) Epothilone A/ γ -CD inclusion complex; (D) DSC (black line) and TG (green line), (a) Epothilone A, (b) β -CD, (c) Epothilone A/ β -CD inclusion complex, (d) γ -CD, and (e) Epothilone A/ γ -CD inclusion complex. (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

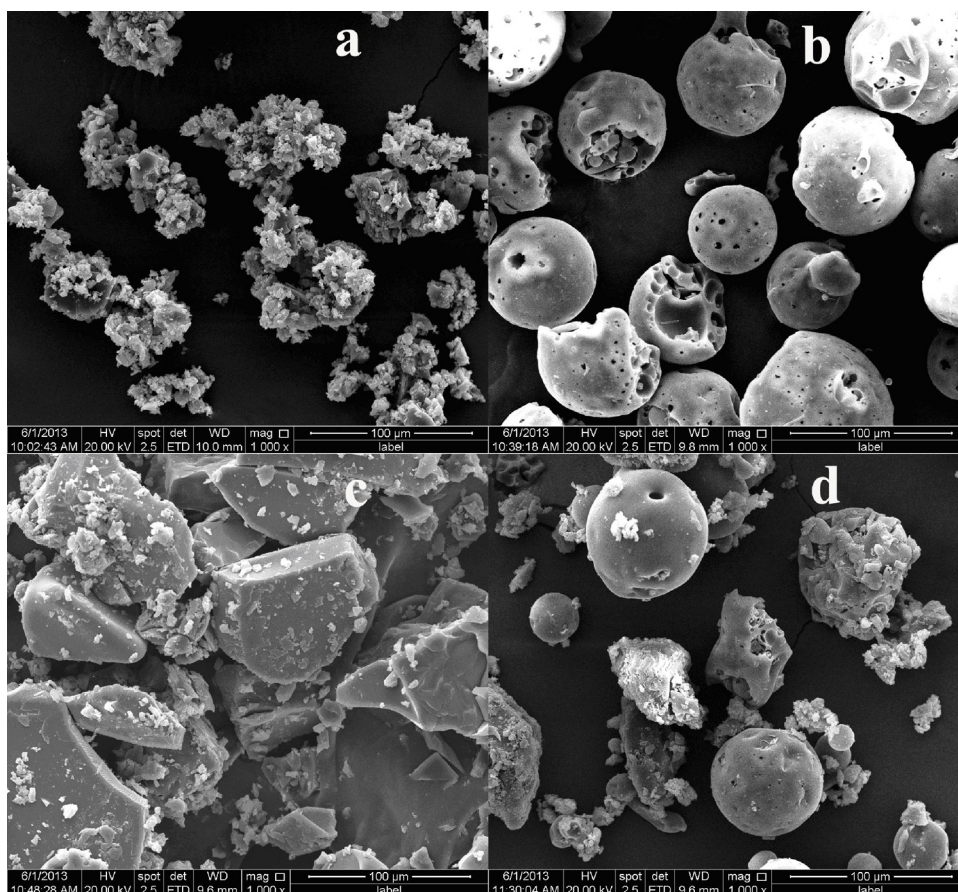


Fig. 6. Scanning electron microphotographs: (a) Epothilone A, (b) HP β CD, (c) Epothilone A/HP β CD inclusion complex, and (d) Epothilone A/HP β CD physical mix.

with the crystals of the free molecules and showed both crystalline components (Fig. 6(d)). In contrast, the Epothilone A/HP β CD inclusion complex appeared in the form of compact and homogeneous plate-like structures with crystal particles in which the original morphology of both components disappeared (Fig. 6(c)). The sizes and shapes of Epothilone A and HP β CD particles were different from those of the inclusion complex, which may indicate inclusion complex formation from the processing of Epothilone A and HP β CD by rotary evaporation. In similar tests, β -CD appeared in a polyhedral form with large dimensions and γ -CD appeared in a polyhedral form with small dimensions. The Epothilone A/ β -CD and Epothilone A/ γ -CD inclusion complexes appeared to be quite different from the sizes and shapes of HP β CD, γ -CD and Epothilone A, respectively, which is a strong indication of inclusion complex formation (see in the Supplementary data).

3.2.6. Solubilization

The water solubility of the Epothilone A/CD complex was assessed by the preparation of a saturated solution (Montassier, Duchêne, & Poelman, 1997). An excess amount of the complex was placed in 2 mL of water (ca. pH 7.0) and the mixture was stirred for 1 h. After removing the insoluble substance by filtration, the filtrate was evaporated under reduced pressure to dryness and the residue was dosed by the weighing method. The results show that the water solubility of this Epothilone A, compared to that of native Epothilone A (ca. 0.125 mg/mL), was remarkably increased to approximately 5.1, 8.2, and 6.8 mg/mL by the solubilizing effects of β -CD, HP β CD and γ -CD, respectively. In the control experiment, a clear solution was obtained after dissolving the Epothilone A/ β -CD 17.0 (mg), Epothilone A/HP β CD 30.0 (mg), and Epothilone A/ γ -CD 24.7 (mg) complexes, which was equivalent to 5.1, 8.2 and

6.8 mg/mL of Epothilone A, respectively, in 1 mL of water at room temperature. This confirmed the reliability of the obtained satisfactory water solubility of the Epothilone A/CD complex, which will be beneficial for the medical utilization of this compound.

3.2.7. Stability

In order to evaluate the stability of Epothilone A/CDs, we tracked the absorbance changes in Epothilone A, Epothilone A/ β -CD, Epothilone A/HP β CD, and Epothilone A/ γ -CD in different buffer solutions, such as physiological saline (0.9% NaCl) or a simulated alkaline body fluid (ca. pH 7.4 $\text{H}_3\text{BO}_3/\text{Na}_2\text{B}_4\text{O}_7/\text{Water}$ buffer system). Solid Epothilone A, Epothilone A/ β -CD, Epothilone A/HP β CD, and Epothilone A/ γ -CD were dissolved quickly and thoroughly and kept at room temperature, and the absorbance was analyzed at 248 nm by the UV/vis spectra every 25 min. Fig. 7(a) illustrates the trend in the relative absorbance A/A_0 (A is the absorbance at the recording time and A_0 is the original absorbance) of Epothilone A and the Epothilone A/CD complexes in 0.9% NaCl or pH 7.4 with an interval of 25 min, respectively. At pH 7.4, the relative absorbance of Epothilone A was changed with a faster rate before 225 min, but the relative absorbance of Epothilone A/CDs increased at a slower rate than that of free Epothilone A at the same time point. After 1425 min, the relative absorbance of the free drug and induced complexes reached equilibrium, and the changes in the relative absorbance were about 60%, 15%, 3%, and 10% for Epothilone A, Epothilone A/ β -CD, Epothilone A/HP β CD, and Epothilone A/ γ -CD, respectively. In 0.9% NaCl, a similar phenomenon was observed (Fig. 7(b)), as the relative absorbance of Epothilone A changed faster than that of the Epothilone A/CD complexes. After 1425 min, the changes in the relative absorbance were about 60%, 15%, 10%, and 6% for Epothilone A, Epothilone A/ β -CD, Epothilone A/HP β CD,

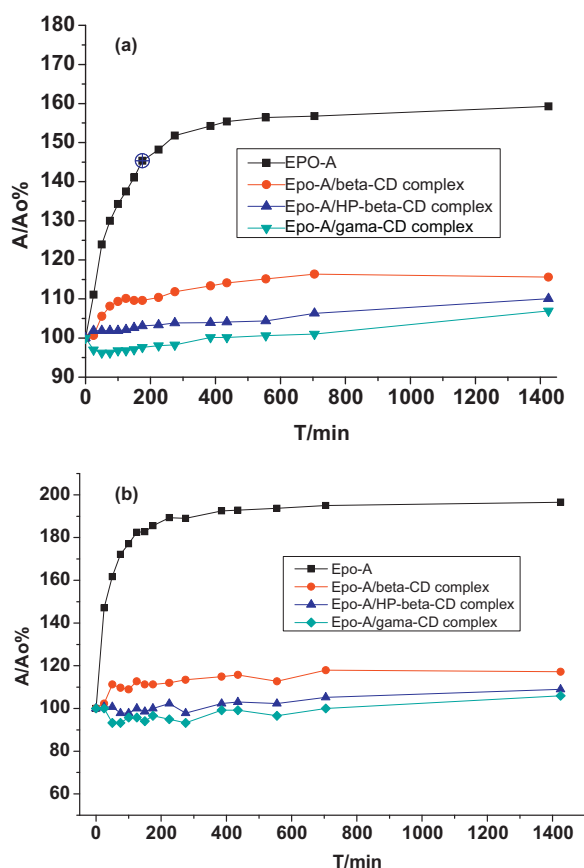


Fig. 7. Degradation profiles of Epothilone A decomposition, (a) in pH 7.4 aqueous solution; and (b) in 0.9 NaCl aqueous solution.

and Epothilone A/ γ -CD, respectively. All these results indicate that Epothilone A/CD complexes are much more stable than free Epothilone A at both pH 7.4 and in 0.9% NaCl.

3.2.8. In vitro cytotoxicity

The cytotoxicity of free Epothilone A and Epothilone A complexed with β -CD, HP β -CD and γ -CD against A549 and MCF-7 cells was individually evaluated using the MTT assay (Carmichael, DeGraff, Gazdar, Minna, & Mitchell, 1987; Mosmann, 1983). The percentage inhibition values for the tumor cells are presented in Table 1. The concentration of the test drugs was 0.06 μ mol/mL. The inhibition rates of Epothilone A and the Epothilone A/ β -CD complex were 85.0% and 86.6% for A549 cell and 77.1% and 77.9% for MCF-7 cells. Epothilone A/CD complexes displayed similar cytotoxicity compared to free Epothilone A against A549 and MCF-7 cells, which indicated that the complexes retained the anticancer activity of the free drug.

Table 1
In vitro cytotoxic activities of Epothilone A and Epothilone A/CDs inclusion complex.

	Inhibition percentage of anticancer activity at 0.06 μ M of sample concentration	
	A549	MCF-7
Epothilone A	85.0%	77.1%
Epothilone A/ β -CD	86.6%	77.9%
Epothilone A/ γ -CD	87.4%	72.9%
Epothilone A/HP β -CD	85.3%	72.3%

4. Conclusions

Inclusion complexes of Epothilone A with β -CD, HP β -CD and γ -CD were prepared. The results of UV-vis, NMR, DSC, XRD, and SEM investigations demonstrated that Epothilone A/CDs has different physicochemical characteristics compared to free Epothilone A. The water solubility and stability of Epothilone A were significantly enhanced by inclusion, and the antitumor activity against A549 and MCF-7 cells was retained. Given the limited applications of Epothilone A, and the facile and environmentally friendly preparation of Epothilone A/CD complexes, this inclusion complexation should be regarded as a promising strategy in the design of a novel formulation of Epothilone A as an anticancer therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.carbpol.2013.11.049>.

References

- Altmann, K. H., Pfeiffer, B., Arseniyadis, S., Pratt, B. A., & Nicolaou, K. C. (2007). The chemistry and biology of epothilones – The wheel keeps turning. *ChemMedChem*, 2(4), 396–423.
- Bollag, D. M., McQueney, P. A., Zhu, J., Hensens, O., Koupal, L., Liesch, J., et al. (1995). Epothilones, a new class of microtubule-stabilizing agents with a taxol-like mechanism of action. *Cancer Research*, 55(11), 2325–2333.
- Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D., & Mitchell, J. B. (1987). Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of radiosensitivity. *Cancer Research*, 47(4), 943–946.
- Chen, W., Yang, L., Ma, S., Yang, X., Fan, B., & Lin, J. (2011). Crassicauline A/ β -cyclodextrin host–guest system: Preparation, characterization, inclusion mode, solubilization and stability. *Carbohydrate Polymers*, 84(4), 1321–1328.
- Chen, Y., Xu, J., Yu, H., Qing, C., Zhang, Y., Wang, L., et al. (2008). Cytotoxic phenolics from *Bulbophyllum odoratissimum*. *Food Chemistry*, 107(1), 169–173.
- Davis, M. E., & Brewster, M. E. (2004). Cyclodextrin-based pharmaceuticals: Past, present and future. *Nature Reviews Drug Discovery*, 3(12), 1023–1035.
- de Araujo, D. R., Tsuneda, S. S., Cereda, C. M., Del, G. F. C. F., Prete, P. S., Fernandes, S. A., et al. (2008). Development and pharmacological evaluation of ropivacaine-2-hydroxypropyl- β -cyclodextrin inclusion complex. *European Journal of Pharmaceutical Sciences*, 33(1), 60–71.
- Dimarco, J. D., Gougoutas, J. Z., Vitez, I. M., Davidovich, M., Galella, M., Malloy, T. M., et al. (2002). New crystalline polymorphs of an epothilone analog useful for treating cancer or other proliferative diseases.
- Duchêne, D. (Ed.). (1987). *Cyclodextrins and their industrial uses*. Paris: Editions de Santé.
- Forli, S., Manetti, F., Altmann, K. H., & Botta, M. (2010). Evaluation of novel epothilone analogues by means of a common pharmacophore and a QSAR pseudoreceptor model for taxanes and epothilones. *ChemMedChem*, 5(1), 35–40.
- Gerth, K., Bedorf, N., Hofle, G., Irschik, H., & Reichenbach, H. (1996). Epothilones A and B: Antifungal and cytotoxic compounds from *Sorangium cellulosum* (Myxobacteria). Production, physico-chemical and biological properties. *Journal of Antibiotics* (Tokyo), 49(6), 560–563.
- Higuchi, T., & Connors, K. A. (1965). Phase solubility techniques. *Advances in Analytical Chemistry Instrumentation*, 4, 117–212.
- Höfle, G., Bedorf, N., Steinmetz, H., Schomburg, D., Gerth, K., & Reichenbach, H. (1996). Epothilone A and B—Novel 16-membered macrolides with cytotoxic activity: Isolation, crystal structure, and conformation in solution. *Angewandte Chemie International Edition in English*, 35(13–14), 1567–1569.
- Jiang, H., Zhang, S., Guan, Q., Chen, C., Gao, F., & Zhang, Y. (2007). ^1H NMR investigations of inclusion complexes between β -cyclodextrin and 1-hexadecanol. *Current Drug Discovery Technology*, 4(4), 295–297.
- Karathanos, V. T., Mourtzinos, I., Yannakopoulou, K., & Andrikopoulos, N. K. (2007). Study of the solubility, antioxidant activity and structure of inclusion complex of vanillin with β -cyclodextrin. *Food Chemistry*, 101(2), 652–658.
- Kemelbekov, U., Luo, Y., Orynbekeva, Z., Rustembekov, Z., Haag, R., Saenger, W., et al. (2011). IR, UV and NMR studies of β -cyclodextrin inclusion complexes

- of kazcaine and prosidol bases. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, 69(1–2), 181–190.
- Lechleider, R. J., Kaminskas, E., Jiang, X., Aziz, R., Bullock, J., Kasliwal, R., et al. (2008). Ixabepilone in combination with capecitabine and as monotherapy for treatment of advanced breast cancer refractory to previous chemotherapies. *Clinical Cancer Research*, 14(14), 4378–4384.
- Lee, F. Y., Smykla, R., Johnston, K., Menard, K., McGlinchey, K., Peterson, R. W., et al. (2009). Preclinical efficacy spectrum and pharmacokinetics of ixabepilone. *Cancer Chemotherapy and Pharmacology*, 63(2), 201–212.
- Liu, Y., & Chen, Y. (2006). Cooperative binding and multiple recognition by bridged bis(beta-cyclodextrin)s with functional linkers. *Accounts of Chemical Research*, 39(10), 681–691.
- Loftsson, T., Hreinsdottir, D., & Masson, M. (2005). Evaluation of cyclodextrin solubilization of drugs. *International Journal of Pharmaceutics*, 302(1–2), 18–28.
- Loftsson, T., & Duchene, D. (2007). Cyclodextrins and their pharmaceutical applications. *International Journal of Pharmaceutics*, 329(1–2), 1–11.
- Misiuk, W., & Zalewska, M. (2009). Investigation of inclusion complex of trazodone hydrochloride with hydroxypropyl- β -cyclodextrin. *Carbohydrate Polymers*, 77(3), 482–488.
- Montassier, P., Duchêne, D., & Poelman, M. C. (1997). Inclusion complexes of tretinoin with cyclodextrins. *International Journal of Pharmaceutics*, 153, 199–209.
- Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65(1–2), 55–63.
- Rivkin, A., Chou, T. C., & Danishefsky, S. J. (2005). On the remarkable antitumor properties of fludelone: How we got there. *Angewandte Chemie International Edition in English*, 44(19), 2838–2850.
- Wu, H., Liang, H., Yuan, Q., Wang, T., & Yan, X. (2010). Preparation and stability investigation of the inclusion complex of sulforaphane with hydroxypropyl- β -cyclodextrin. *Carbohydrate Polymers*, 82(3), 613–617.
- Yang, B., Chen, Y., Lin, J., & Liu, Y. (2008). Inclusion complexation behaviors of 3-tigloyl-azadirachtol with β -cyclodextrin derivatives. *Current Pharmaceutical Analysis*, 3(4), 176–182.
- Yang, B., Lin, J., Chen, Y., & Liu, Y. (2009). Artemether/hydroxypropyl-beta-cyclodextrin host-guest system: Characterization, phase-solubility and inclusion mode. *Bioorganic & Medicinal Chemistry*, 17(17), 6311–6317.
- Yang, B., Yang, L., Lin, J., Chen, Y., & Liu, Y. (2009). Binding behaviors of scutellarin with α -, β -, γ -cyclodextrins and their derivatives. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, 64(1–2), 149–155.
- Yang, L. J., Yang, B., Chen, W., Huang, R., Yan, S. J., & Lin, J. (2010). Host-guest system of nimbin and beta-cyclodextrin or its derivatives: Preparation, characterization, inclusion mode, and solubilization. *Journal of Agricultural and Food Chemistry*, 58(15), 8545–8552.
- Yang, L., Chen, W., Ma, S., Gao, Y., Huang, R., Yan, S., et al. (2011). Host-guest system of taxifolin and native cyclodextrin or its derivative: Preparation, characterization, inclusion mode, and solubilization. *Carbohydrate Polymers*, 85(3), 629–637.
- Yang, R., Chen, J., Dai, X., Huang, R., Xiao, C., Gao, Z., et al. (2012). Inclusion complex of GA-13315 with cyclodextrins: Preparation, characterization, inclusion mode and properties. *Carbohydrate Polymers*, 89(1), 89–97.