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Inclusion complexes of azadirachtin with native and methylated cyclodextrins: solubilization and binding ability

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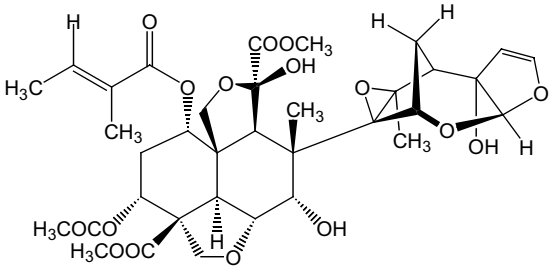
Abstract—The inclusion complexation behavior of azadirachtin with several cyclodextrins and their methylated derivatives has been investigated in both solution and the solid state by means of XRD, TG–DTA, DSC, NMR, and UV–vis spectroscopy. The results show that the water solubility of azadirachtin was obviously increased after resulting inclusion complex with cyclodextrins. Typically, β-cyclodextrin (β-CD), dimethyl-β-cyclodextrin (DMβCD), permethyl-β-cyclodextrin (TMβCD), and hydroxypropyl-β-cyclodextrin (HPβCD) are found to be able to solubilize azadirachtin to high levels up to 2.7, 1.3, 3.5, and 1.6 mg/mL (calculated as azadirachtin), respectively. This satisfactory water solubility and high thermal stability of the cyclodextrin–azadirachtin complexes, will be potentially useful for their application as herbal medicine or healthcare products.

1. Introduction

The neem tree, *Azadirzchta indica*, is a tropical plant that is well known for its pesticidal properties.[1,2] Many studies have demonstrated that its seed contains abundant limonoids and simple terpenoids that are responsible for its biological activity.[3–7] Among these limonoids, azadirachtin A (commonly referred to as azadirachtin, Chart 1) is considered to be the most important active principle due to its various effects on insects,[8] and has gained considerable attentions as a potential nontoxic, biodegradable, and natural pesticide.[9] Furthermore, azadirachtin also has great application in herbal medicine/healthcare products, especially for major skin diseases, anti-malarial, anti-tuberculosis, anti-worms, anti-clotting, blood detoxifier, anti-viral, anti-periodontitic, anti-bacterial, anti-fungal, etc.[10] For example, cytotoxicity of azadirachtin in human glioblastoma cell lines has been investigated by Akudugu et al.,[11] which indicates that azadirachtin can affect reproductive integrity and cell division. However, the utility of azadirachtin as biopesticide or herbal medicine is greatly limited by its low water solubility and instability as a result of the propensity to undergo complicated, irreversible rearrangements under mild acidic, basic, and photolytic conditions.[12,13] In the past decades, many efforts have been contributed to the syntheses of new azadirachtin derivatives and their structure–bioactivity relationship[14] to overcome the difficulties mentioned above. Unfortunately, the methods for the selective modification of azadirachtin usually involve lots of protection and deprotection steps and/or complicated chromatographic separations. Considering these limitations, an easy and promising approach for a better formulation of azadirachtin is to improve the water solubility and stability of azadirachtin by introducing some nontoxic solubilizers. For example, Li and Wu[15] and Xu[16] have reported the preparation of azadirachtin-containing microemul-



Azadirachtin

Chart 1.

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sion with some additives, such as pyrethrin and rotenone. However, these azadirachtin-containing microemulsion systems can be only dispersed, but not dissolved, in water. Among the various inorganic and organic compounds generally used as drug carriers, cyclodextrins (CDs), a class of biodegradable cyclic oligosaccharides mainly with six to eight D-glucose units linked by α-1,4-glucose bonds, are able to encapsulate various organic guests within their hydrophobic cavities to afford host–guest complex or supramolecular species in aqueous solution.[17] This fascinating property enables them to be successfully utilized as drug carriers,[18–20] separation reagents,[21] enzyme mimics,[22] and photochemical sensors,[23] etc. Recently, some patents about the inclusion complex formation between CDs and azadirachtin have been reported.[24] However, to the best of our knowledge, the comprehensive investigations about the inclusion behaviors and stabilities of CD/azadirachtin complexes are still rare. More recently, we reported that oligoethylenediamine bridged bis(β-CD) could form 2:1 inclusion complex with palictaxel, which significantly enhanced the water solubility of palictaxel from ca. 0 to 2 mg/mL.[25] Herein, we wish to report the preparation and characterization of some water-soluble inclusion complexes formed by azadirachtin and CDs (Chart 2). It is our special interest to explore the solubilization effect of CDs toward azadirachtin and the binding ability of the resultant inclusion complexes, which will provide a useful approach to achieve novel azadirachtin-based healthcare products with high water solubility, high bioactivity, and low toxicity.

2. Results and discussion

2.1. Spectral titration

Quantitative investigation of the inclusion complexation behavior of α-, β-, and γ-CDs with azadirachtin are examined in a water/glycerin (v:v = 4:1) mixed solution by means of spectrophotometric titration method owing to the rather low water solubility of azadirachtin. As illustrated in Figure 1, the absorbance intensity of azadirachtin gradually is increased with the stepwise addition of β-CD. In the control experiment, the pH value of the solution does not change appreciably during the experimental procedure. These results indicate that the binding behavior is mainly dependent on the individual structural features of host and guest.

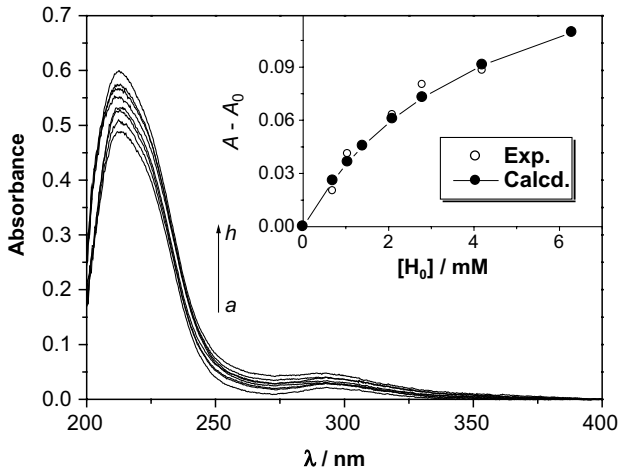

Figure 1. Absorption spectral changes of azadirachtin (0.5 mM) upon addition of host β-CD (0–6.3 mM from a to h) in a water/glycerin (v:v = 4:1) mixed solution and the nonlinear least squares analysis (inset) of the differential intensity (ΔA at 213 nm) to calculate the complex stability constant (K_s).

Using a nonlinear least squares curve-fitting method,[26] we obtained the complex stability constant for each host–guest combination. Figure 1 (inset) illustrates a typical curve-fitting plot for the titration of azadirachtin with β-CD, which shows the excellent fits between the experimental and calculated data. In the repeated measurements, the K_s values are reproducible within an error of ±5%. The stability constant (K_s) and Gibbs free energy change ($-\Delta G^0$) for the inclusion complexation of CDs with azadirachtin are listed in Table 1.

2.2. Binding ability

Extensive studies have revealed that the size/shape-fit concept plays a crucial role in the formation of inclusion complexes of host CDs with guest molecules of various structures. On the basis of this concept, several weak

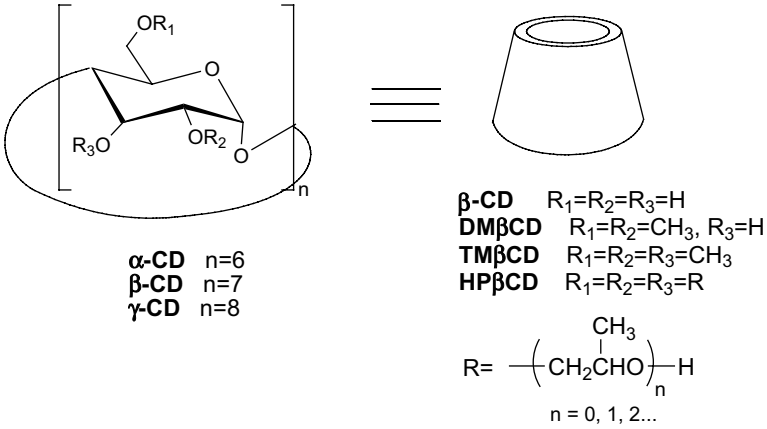

Chart 2.

Table 1. Complex stability constant (K_s) and Gibbs free energy change ($-\Delta G^0$) for the inclusion complexation of native CDs with azadirachtin guest in a water/glycerin (v:v = 4:1) mixed solution

Host	K_s/M^{-1}	$\log K_s$	$-\Delta G°/kJ\ mol^{-1}$
α-CD	—[a]	—	—
β-CD	238	2.38	13.6
γ-CD	73	1.86	10.6

[a] The spectral changes of α-CD with azadirachtin are too small to calculate stability constant.

intermolecular forces such as ion–dipole, dipole–dipole, van der Waals, electrostatic, hydrogen bond, and hydrophobic interactions are known to cooperatively contribute to the inclusion complexation. It is well known that each of α-, β-, and γ-CDs possesses a cyclic truncated-cone cavity with a height of 0.79 nm, but their average inner diameters are 0.50, 0.62, and 0.79 nm for α-, β-, and γ-CDs, respectively. Therefore, the host–guest size matching may dominate the stability of the complexes formed between these CDs and azadirachtin. From Table 1, we can see that β-CD, which possessed a moderate cavity size, can better complex with the guest azadirachtin, giving the stronger K_s value than α- and γ-CDs. In addition, it is demonstrated that the modified derivatives of CDs usually show the higher solubilization effect and the stronger binding ability toward model substrates than native CDs. Therefore, we also select heptakis-(2,6-tri-*O*-methyl)-β-CD (DMβCD), heptakis(2,3,6-tri-*O*-methyl)-β-CD (TMβCD), and hydroxypropyl-β-cyclodextrin (HPβCD) as host molecules to investigate their inclusion complexation abilities toward azadirachtin. Unfortunately, due to the absorption disturbance of DMβCD, TMβCD, and HPβCD in the wavelength range of 200–300 nm, the K_s value of these CDs with azadirachtin cannot be obtained. However, the NMR, TG–DTA, DSC, and XRD examinations still demonstrate that these three CD derivatives can efficiently associate with azadirachtin to form the inclusion complex, which will be described below.

2.3. NMR analysis

In order to explore the possible inclusion mode of CD–azadirachtin complexes, we compare the ^{1}H NMR spectra of azadirachtin in the presence of host CDs (Fig. 2). Owing to its poor water solubility, azadirachtin is transparent to ^{1}H NMR under most conditions when D_2O is used as solvent. Assessment of the azadirachtin complexes by ^{1}H NMR clearly demonstrates the presence of the framework protons of azadirachtin molecule consistent with the significant solubilization. As illustrated in Figure 2, a majority of azadirachtin protons (22H) display the chemical shifts at δ 1.0–2.5 ppm, which are distinct from the CD protons. By comparing the integration area of these protons with that of the CD's H-1 protons, we can calculate the inclusion stoichiometry of CD–azadirachtin complexes, that is, 1:1 for β-CD–azadirachtin complexes and 2:1 for TMβCD–azadirachtin complex. In addition, the 2:1 inclusion stoichiometry is also observed in the cases of DMβCD–azadirachtin and HPβCD–azadirachtin complexes.

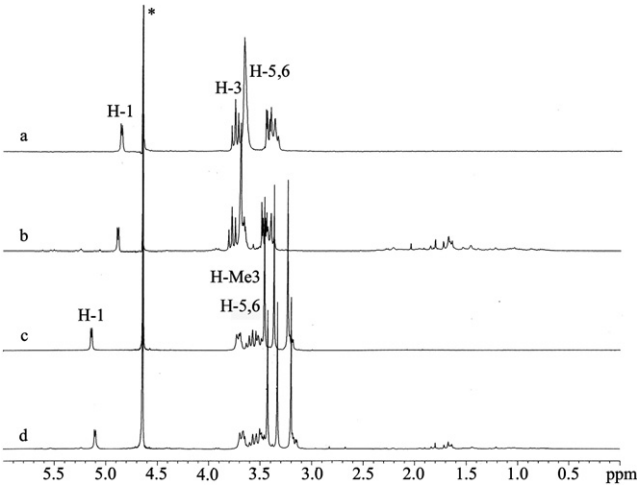

Figure 2. ^{1}H NMR spectra of azadirachtin in the absence and presence of β-CD and TMβCD in D_2O at 25 °C, respectively. (a) β-CD, (b) β-CD–azadirachtin complex, (c) TMβCD, (d) TMβCD–azadirachtin complex (asterisk highlights the water peak).

To further explore the inclusion mode, the chemical shifts of CD protons in the absence and presence of azadirachtin are listed in Table 2. As can be seen from Table 2, after inclusion complexation with azadirachtin, a negligible effect is observed on the δ values of H-4 and H-5 protons of β-CD. In contrast, those values of H-1, H-2, H-3, and H-6 protons exhibit the relatively weak but significant changes (0.03–0.04 ppm). It is fairly noteworthy that H-3 protons shift ca. 0.03 ppm, but

Table 2. The chemical shifts (δ) of β-CD, TMβCD, β-CD–azadirachtin and TMβCD–azadirachtin complexes

		δ (ppm)			
		β-CD	β-CD–azadirachtin complex	TMβCD	TMβCD–azadirachtin complex
H-1	d	4.85	4.89	5.15	5.11
H-2	dd	3.45	3.49	3.20	3.16
H-3	dd	3.78	3.81	3.53	3.50
H-4	dd	3.40	3.40	3.56	3.53
H-5	m	3.66	3.66	3.70	3.65
H-6	dd	3.66	3.69	3.52	3.46
H-Me2	s	—	—	3.37	3.33
H-Me3	s	—	—	3.46	3.43
H-Me6	s	—	—	3.24	3.20

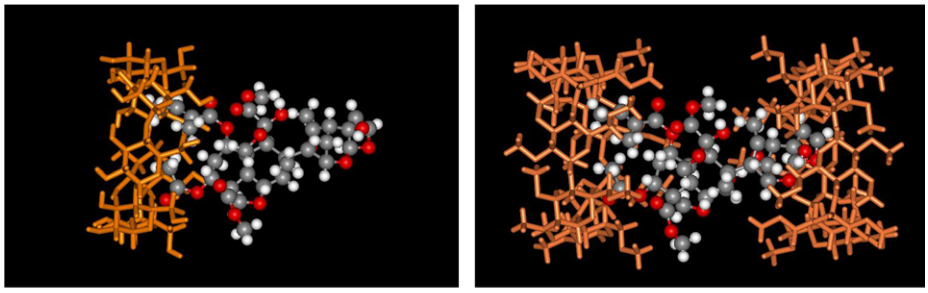

Figure 3. Possible inclusion modes of β-CD–azadirachtin and TMβCD–azadirachtin complexes.

H-5 protons show no appreciable shifts after resulting inclusion complex. Because both H-3 and H-5 protons are located in the interior of CD cavity, and H-3 protons are near the wide side of cavity while H-5 protons near the narrow side, this phenomenon may indicate that azadirachtin should penetrate into the CD cavity from the wide side. In contrast, all of the TMβCD protons show the appreciable shifts after inclusion complexation with azadirachtin (0.03–0.06 ppm). By comparing these chemical shifts, we can find the shifts of H-5 (0.05 ppm) and H-6 (0.07 ppm) protons are larger than those of H-3 protons (0.03 ppm), indicating that azadirachtin may enter the cavity of TMβCD from the narrow side. Based on these observations, together with the 1:1 and 2:1 stoichiometry, we can deduce the possible inclusion modes of azadirachtin with CDs as illustrated in Figure 3.

2.4. XRD analysis

The powder X-ray diffraction (XRD) patterns of azadirachtin, β-CD, TMβCD as well as their inclusion complexes are illustrated in Figure 4. As can be recognized from Figure 4, azadirachtin is amorphous (Fig. 4a), but β-CD (Fig. 4b) and TMβCD (Fig. 4c) are in crystalline form. In contrast, the XRD of β-CD–azadirachtin and TMβCD–azadirachtin complexes (Fig. 4d and e) exhibit the halo patterns, which are quite different from a superimposition of crystalline β-CD (or TMβCD) and the amorphous azadirachtin, indicating the formation of the inclusion complex between β-CD (or TMβCD) and azadirachtin. In addition, most of the crystalline diffraction peaks of β-CD or TMβCD disappear after complexation with azadirachtin, indicating that the complexation of azadirachtin makes CDs reorient to some extent. Moreover, less sharp peaks in the XRD of TMβCD–azadirachtin complex may infer that TMβCD–azadirachtin possesses a more amorphous structure than β-CD–azadirachtin complex.

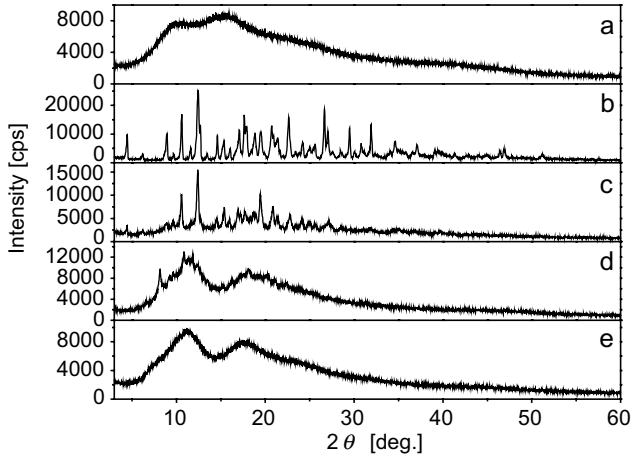

Figure 4. XRD patterns of (a) azadirachtin, (b) β-CD, (c) TMβCD, (d) β-CD–azadirachtin complex, and (e) TMβCD–azadirachtin complex.

2.5. Thermal analysis

The thermal properties of β-CD–azadirachtin and TMβCD–azadirachtin complexes are investigated by thermogravimetric (TG) and differential thermal analysis (DTA) (see Supporting information). A systemic analysis on the TG curves shows that azadirachtin decomposes at ca. 300 °C while β-CD at ca. 200 °C and TMβCD at ca. 170 °C. However, their inclusion complexes show much different thermal stability, that is, the decomposition temperatures are ca. 240 and 250 °C for β-CD–azadirachtin and TMβCD–azadirachtin complexes, respectively. On the other hand, the DTA curve of β-CD–azadirachtin or TMβCD–azadirachtin complex displays a peak at ca. 368 or 341 °C, respectively, corresponding to the dissociating temperature of pristine azadirachtin (that value for free azadirachtin is 349 °C). This phenomenon indicates that the potent healthcare ingredient azadirachtin does not change its typical thermal property after inclusion complexation.

Furthermore, the differential scanning calorimetry (DSC) thermogram gives the further information about the thermal property of β-CD–azadirachtin and TMβCD–azadirachtin complexes. As shown in Figure 5, the DSC curve of azadirachtin displays two endothermic peaks at 154 and 200 °C. In contrast, the DSC curve of pristine β-CD or TMβCD shows an endothermic peak at 101 or 155 °C, respectively. However, we can find that, in the DSC curves of CD–azadirachtin complexes, the endothermic peaks at about 154 and 200 °C corresponding to the free azadirachtin disappear, along with the appearance of a new exothermic peak at 97 °C (or 63 °C) in the case of β-CD–azadirachtin(or TMβCD–azadirachtin) system, showing the β-CD–

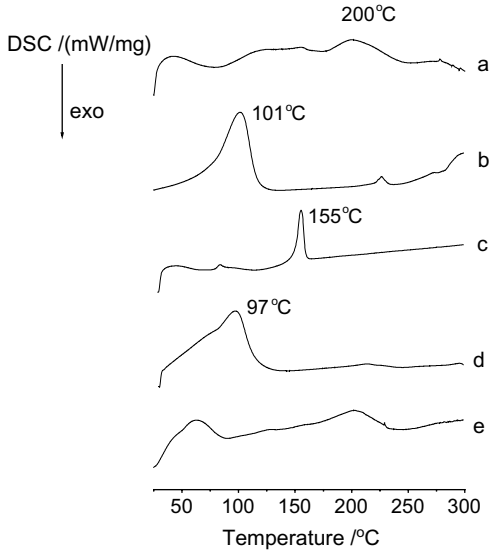

Figure 5. DSC diagrams of (a) azadirachtin, (b) β-CD, (c) TMβCD (d) β-CD–azadirachtin, and (e) TMβCD–azadirachtin complex.

azadirachtin complex is rather stable than TMβCD–azadirachtin complex. In similar tests, DMβCD–azadirachtin or HPβCD–azadirachtin complex also show a high decomposition temperature up to 220 or 250 °C, respectively. All these samples have been analyzed by HPLC confirming the thermal degradation occurrence. These results not only further confirm the formation of CD–azadirachtin complexes, but also indicate that the four resultant CD–azadirachtin complexes start to decompose only at a temperature above 220 °C, which means that these complexes are fairly stable in thermal viewpoint.

2.6. Water solubility

The water solubility of CD–azadirachtin complex is assessed by the preparation of its saturated solution.[27] Excess amount of complex is put into 2 mL of water (ca. pH 6.0) and the mixture is stirred for 1 h. After removing the insoluble substance by filtration, the filtrate is evaporated under reduced pressure to dryness and the residue is dosed by weighing method. The results show that the water solubility of β-CD–azadirachtin, DMβCD–azadirachtin, TMβCD–azadirachtin, and HPβCD–azadirachtin complexes, comparing with that of azadirachtin (50 μg/mL[28]), is dramatically increased to approximately 2.7, 1.3, 3.5, and 1.6 mg/mL (calculated as azadirachtin residue), respectively. In the control experiment, a clear solution is obtained after dissolving β-CD–azadirachtin (14 mg), DMβCD–azadirachtin (10.5 mg), TMβCD–azadirachtin (17.4 mg), or HPβCD–azadirachtin (14 mg) complex, respectively, which is equivalent to 2.7, 1.3, 3.5, or 1.6 mg of azadirachtin, in 1 mL of water at room temperature. This subsequently confirms the reliability of the obtained satisfactory water solubility of CD–azadirachtin complexes, which will be beneficial to the medical utilization of this compound.

3. Experimental

3.1. Materials

Azadirachtin (32.3%) used in this work was obtained by microwave assisted extraction from neem dry leaf in Yunnan Province, PR China. α-, β-, γ-CDs, heptakis-(2,6-tri-*O*-methyl)-β-CD (DMβCD), and hydroxypropyl-β-cyclodextrin (HPβCD) were commercially available and used without further purification. Heptakis(2,3,6-tri-*O*-methyl)-β-CD (TMβCD) was prepared according to the reported procedures.[29]

3.2. Measurements

NMR experiments were performed on a Varian Mercury VX300 spectrometer at 298 K in a deuterium oxide solution. Tetramethylsilane was used as reference and no correction was made for susceptibility of the capillary. Thermogravimetric (TG) and differential thermal analysis (DTA) were recorded with a RIGAKU Standard type. Samples were heated at 10 °C/min from room temperature to 500 °C in a dynamic nitrogen atmosphere (flow rate = 70 mL/min). Powder X-ray diffraction (XRD) patterns were obtained using a Rigaku D/max-2500 diffractometer with Cu Kα radiation (40 kV, 100 mA). Powder samples were mounted on a sample holder and scanned with a step size of $2\theta = 0.02°$ between $2\theta = 3°$ and 35°. Differential scanning calorimetry (DSC) was performed with a NETZSCH DSC 204 instrument with a heating rate of 10 °C/min from 26 to 300 °C with a heating rate of 10 K/min. UV/Vis spectra were performed on a Shimadzu UV 2401 spectrophotometer. Considering the poor water solubility of azadirachtin, a water–glycerin (v:v = 4:1) was used in the spectral measurements.

3.3. Preparation of β-CD–azadirachtin complex

Azadirachtin (0.03 mM, 21.6 mg) and β-CD (0.01 mM, 12.6 mg) were completely dissolved in a mixed solution of ethanol and water (ca. 7 mL, v:v = 1:5), and the mixture was stirred for 4 days at room temperature. After evaporating the ethanol from the reaction mixture, the uncomplexed azadirachtin was removed by filtration. The filtrate was evaporated under the reduced pressure to remove the solvent and dried in vacuum to give β-CD–azadirachtin complex (22.6 mg, yield 92%). ^{1}H NMR (300 MHz, D_2O, TMS): δ 0.6–2.42 (m, 22H, azadirachtin protons), 3.36–3.80 (m, >50H, H-2–6 of β-CD and some protons of azadirachtin), 4.87–4.89 (s, 7H, H-1 of β-CD).

3.4. Preparation of DMβCD/azadirachtin complex

DMβCD–azadirachtin complex was similarly prepared in ca. 80% yield from TMβCD and azadirachtin. ^{1}H NMR (300 MHz, D_2O, TMS): δ 0.6–2.4 (m, 22H azadirachtin protons), 3.14–3.70 (m, >200H, H-2–6 and OCH_3-2, 6 of TMβCD and some protons of azadirachtin), 5.10–5.11 (s, 14H, H-1 of DMβCD).

3.5. Preparation of TMβCD/azadirachtin complex

TMβCD–azadirachtin complex was similarly prepared in ca. 85% yield from TMβCD and azadirachtin. ^{1}H NMR (300 MHz, D_2O, TMS): δ 0.6–2.4 (m, 22H azadirachtin protons), 3.14–3.70 (m, >220H, H-2–6 and OCH_3-2, 3, 6 of TMβCD and some protons of azadirachtin), 5.10–5.11 (s, 14H, H-1 of TMβCD).

3.6. Preparation of HPβCD/azadirachtin complex

HPβCD–azadirachtin complex was similarly prepared in ca. 90% yield from HPβCD and azadirachtin. ^{1}H NMR (300 MHz, D_2O, TMS): δ 0.6–2.4 (m, 22H azadirachtin protons), 3.14–3.70 (m, >220H, H-2–6 and CH_2- and CH_3-2, 3, 6 of HPβCD and some protons of azadirachtin), 5.10–5.11 (s, 14H, H-1 of HPβCD).

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2005.03.051.

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