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Synthesis of a new water-soluble phototherapeutic sensitizer from hypocrellin B with enhanced red absorption

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Abstract

Hypocrellins are new photodynamic agents. To improve the red absorption and water-solubility of these new type photosensitizers, we have synthesized a new hypocrellin derivative, i.e. di-cysteine substituted hypocrellin B (DCHB), using the photoreaction of hypocrellin B with cysteine in an aerated ethanol-buffer (1:3 by volume, pH > 10) solution. Thiylation, followed by amination, afforded this new compound. The yield of DCHB is significantly influenced by the oxygen content, the pH value and the solvent used in the reaction. Compound DCHB is completely water-soluble, and its longer absorption band shifts to 684 nm, failing in the domain of the phototherapeutic window (600–900 nm). Moreover, DCHB is photodynamically active in terms of both the type I and type II mechanisms. These observations confirm that DCHB is a promising and potential phototherapeutic agent.

Keywords: Hypocrellin B; Cysteine; Amination; Thiylation; Phototherapeutic agent

1. Introduction

Photodynamic therapy (PDT), using red light and sensitizers of the porphyrin family, is a promising new treatment for light-accessible tumors [1,2]. The initial photochemical processes leading to cell death may follow two principal pathways, viz., upon light absorption, the photosensitizer transfers the energy to O_2 to yield singlet oxygen ($^1O_2$), a potent oxidizer, or, alternatively engages in charge transfer reactions with biomolecules [3]. Either pathway requires oxygen to propagate the damage via radical chain reactions. Most clinical applications of PDT use Photofrin®, which consists of a mixture of hematoporphyrin derivatives [4]. Several properties of Photofrin, including undesirable side effects, could be improved upon and much work has been devoted to develop new photosensitizers [5–7]. Among the latter, hypocrellins, natural perylenequinonoid pigments, including hypocrellin A (HA) and hypocrellin B (HB), have been proposed as potential second-generation photosensitizers for PDT because of their high quantum yields of singlet oxygen, substantial absorption in the red spectral region, availability in pure monomeric form, and facility for site-directed chemical modifications to optimize properties of red absorption, water solubility, tissue distribution and toxicity [8].

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In the past, perylenequinonoid compounds have been used as phototherapeutic agents for various skin diseases and superfacial tumors, and have been taken orally as folk medicine in China [9,10]. Recently, hypocrellins have been reported to display photoinduced antiviral activities against the human immunodeficiency virus (HIV-1), herpes simplex virus Type I, Sindbis virus, and vesicular stomatitis (VSV) [11–14]. Zou and coworkers have investigated the damage of pBR322 DNA by HA in liposomes and its derivative in solution [15]. All of these results show that hypocrellin dyes are new potential photosensitizers.

However. the naturally-occurred hypocrellins are not water-soluble and do not exhibit strong red absorption at wavelength longer than 600 nm. Many efforts have been made to improve these two aspects. Diwu et al. have synthesized amino-substituted hypocrellin derivatives [16,17]. The most promising results have been shown with these amino-substituted hypocrellins both in vitro and in vivo [18,19]. Mono-cysteine substituted hypocrellin B (MCHB) has been synthesized thermally [20,21] and photochemically [22], and characterised [20–22]. The red absorption and water-solubility of MCHB are significantly enhanced with respect to those of HB. Further investigations on the photoreaction of HB with cysteine indicate that a new and completely water-soluble product can be obtained by improving the reaction process. The yield of this new derivative, di-cysteine substituted hypocrellin B (DCHB), is sensitive to oxygen content, pH value and the organic solvent used in the reaction.

2. Results and discussion

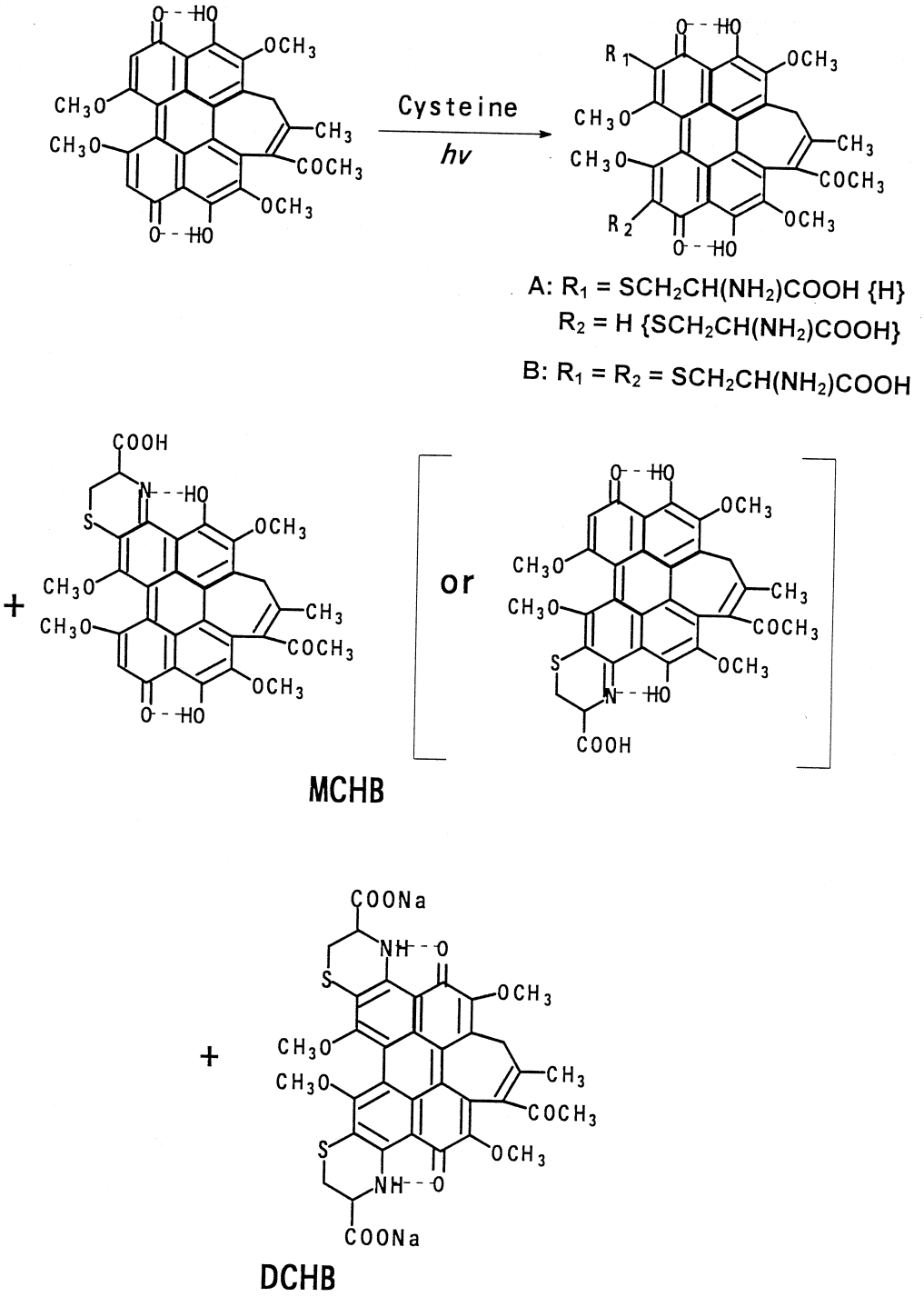
2.1. Synthesis of new dye

Irradiation of HB (5×10^{-4} M) and cysteine (0.1 M) in an aerated ethanol-buffer (1:3 by volume, pH > 10) solution with light of wavelength longer than 470 nm afforded the following products: A, B, MCHB and DCHB. The reaction is demonstrated in Scheme 1.

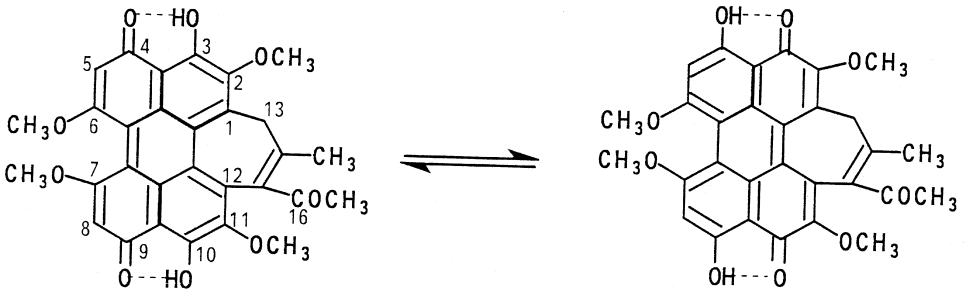
According to the chemical structure of HB (Fig. 1), there are four types of positions expected to be reactive towards cysteine, i.e. (i) the aromatic ring (positions 5 and 8), (ii) the carbonyl groups (positions 4 and 9; in I), (iii) the side ring, and (iv) positions 2 and 11 [23]. As in the case of other perylenequinonoid pigments, HB exists in an equilibrium of two tautomers (structures I and II in Fig. 1) at ambient temperature. These tautomers have been identified and studied by NMR, EPR and circular dichorism methods [24]. It has been shown by the reaction of HB with either aldehydes or ketones that the several α-active hydrogens on the side ring of HB are relatively deactivated because of the steric hindrance in the ring. As expected, the aromatic ring (positions 5 and 8) in HB is more reactive to thiol compounds than the α-active hydrogens in the side ring of HB; the carbonyl groups (positions 4 and 9 in I) are more reactive to amino group in cysteine than the side acetyl group (position 16) in HB. Both thiol group and amino group exist in cysteine, so dehydration after nucleophilic addition of the thiol group on position 5 or/and 8 might occur between amino group(s) and carbonyl group(s) in A and B to afford cyclic structures, i.e. MCHB and DCHB, respectively.

Compounds A, B and MCHB have been characterized [22]. Compounds A and MCHB both consist of two structural isomers (Scheme 1), respectively, which cannot be separated by thin-layer chromatography (TLC) or even high pressure liquid chromatography (HPLC) [21,22]. The above compounds are soluble both in organic solvents, such as chloroform, ethyl acetate, dimethylsulfoxide (DMSO) and *N,N*-dimethylformamide (DMF), and slightly soluble in water. However, the new compound DCHB is different from all the other products in Scheme 1, being readily soluble in water and slightly soluble in DMSO and methanol.

On the basis of spectral data and specific tests, compound DCHB can be identified to be of the structure shown in Scheme 1. Its molecular ion peak (m/z) appeared at 774 in the mass spectrum, 8 mass units more than that of compound B (m/z 766) [22]. In the ^{1}H-NMR spectrum, there should be two aromatic protons for HB (H at positions 5 and 8), but in fact, for compound DCHB, no aromatic proton is observed. In the IR spectrum


Cysteine
hv
A: R1 = SCH2CH(NH2)COOH {H}
R2 = H {SCH2CH(NH2)COOH}
B: R1 = R2 = SCH2CH(NH2)COOH
or
MCHB
DCHB

Scheme 1.



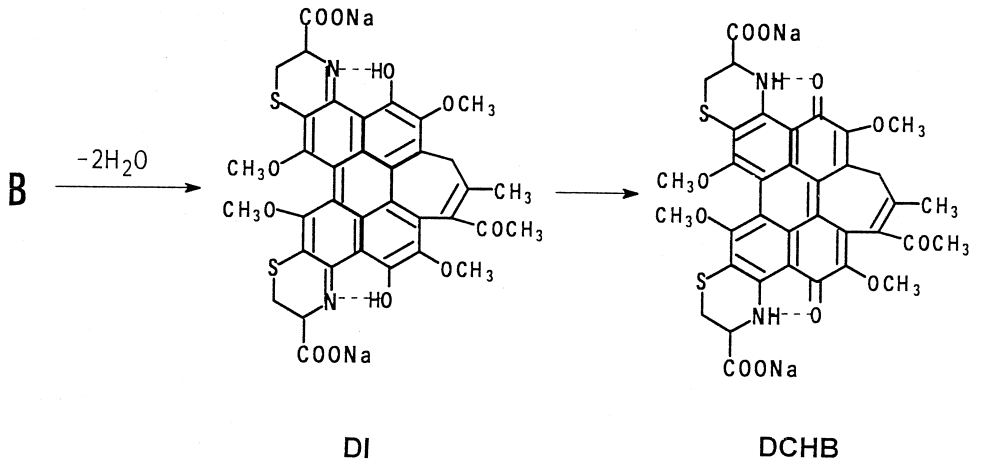
I II

Fig. 1. The tautomerization equilibrium of HB.

of compound DCHB, one strong band at 1599 cm^{-1} of the quinonoid carbonyl groups is observed, and the band at 1284 cm^{-1} may arise from the aromatic C–N group. The IR peaks of the carboxyl group (COOH) cannot be observed, but two bands at 1526 and 1398 cm^{-1} for the carboxylate anion (COO^-) appear, indicating that −COOH exists in the form of sodium carboxylate (COONa), considering that sodium ions are present in the reaction media. The presence of −COONa may be responsible for the excellent water-solubility of DCHB, more than that of any other previously reported hypocrellin derivatives. With respect to the mass spectrum, considering the existence of COONa other than COOH, the molecular ion peak (m/z) of DCHB at 774 (M^+) will be 730 if COONa is replaced by COOH, and it is evident that 730 is 36 mass units less than that of compound B (M^+ 766) [22]. This indicates that elimination of two water molecules between $-NH_2$ and $-C=O$ in compound B probably occurs. This is in a good agreement with an earlier report [25]. The interaction between a simple quinone and cysteamine could yield a monosubsituted quinone, which could then further lose a water molecule between the amino group in the cysteamine and the quinonoid carbonyl group to form a cyclo product [25]. In addition, we have surveyed the effect of pH value on the absorption spectra of DCHB in the pH range between −0.6 and 14. When the pH changes between 7 and 14, the absorption spectrum of DCHB does not change, indicating no phenolic hydroxyl is present in DCHB. When different concentrations of hydrochloric acid are added, the absorption spectrum of DCHB changes significantly (Fig. 3), showing the probable existence of an aromatic amino group. These findings, together with the observation of a strong IR peak for a quinonoid carbonyl group, suggest that the structure of DCHB is consistent with a peri-aminoperylenequinone, but not with a Schiff base as in MCHB. This implies that the first formed intermediate (DI), with a Schiff base structure, then transforms into the final product (DCHB) with a peri-aminoperylenequinone structure (Scheme 2), and the absorption of DCHB is significantly shifted red compared to that of HB (Fig. 2). The good water-solubility and red absorption make DCHB a promising and useful PDT agent.

2.2. Reaction mechanism

We have demonstrated that the photoreactions of HB with thiol compounds (RSH) are consistent with nucleophilic anion addition of Michael type in which RS^- attacks at the 5 or/and 8 positions in HB followed by oxidation [22]; compounds A and B are thus formed. The addition of α-phenyl-*N*-*tert*-butyl nitrone (PBN), a good spin trap for a thiyl radical (RS•), has no effect, ruling out the partipation of RS• in the formation of products [22]. Since there are two reactive groups in cysteine, i.e. $-NH_2$ as well as −SH, we carried out experiments to survey the reaction pathway in the formation of DCHB, and the influence of oxygen, pH value and solvent on the yield of DCHB.



Scheme 2.

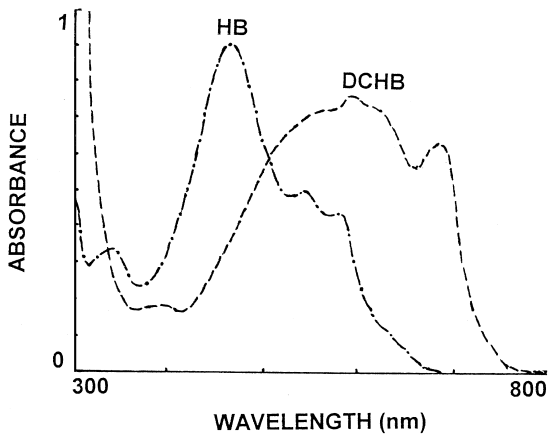


Fig. 2. Absorption spectra of HB (in chloroform) and DCHB (in water).

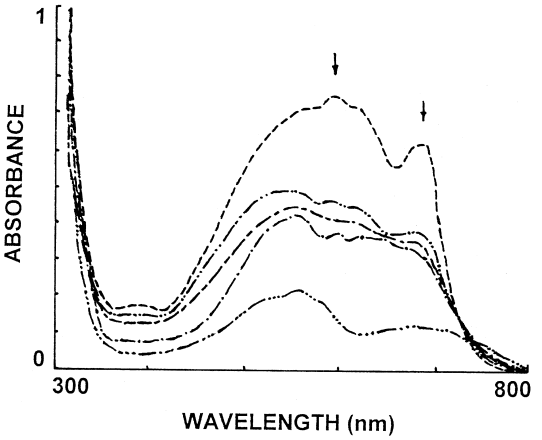


Fig. 3. Absorption spectrum changes of DCHB in aqueous solution when different concentrations of hydrochloric acid (HCl) are added: 0, 0.25, 0.5, 2.0, 4.0 M.

When an aerated ethanol-buffer (1;3 by volume, pH > 10) solution of HB (5×10^{-4} M) and aminoacetic acid (H_2NCH_2COOH) (0.2 M) is irradiated with light of wavelength longer than 470 nm or left in the dark, no reaction occurs. This indicates that amination in the photoreaction of HB with cysteine does not come from the direct reaction of $-NH_2$ in cysteine and $-C=O$ in HB. Significantly, the yield of DCHB rapidly increases when the aerated reaction solution is left aside in the dark after 20 min irradiation. However, the yield of DCHB decreases if the reaction solution irradiated for 20 min continues to be irradiated. These findings suggest that amination follows the thiylation of HB, and proceeds via a thermal reaction, i.e. thiylation of HB facilitates the amination that occurs after thiylation. The observation that the yield of DCHB decreases when the irradiation time is over 20 min might result from the photoinduced degradation of DCHB in strong alkaline and aerated media. However, DCHB is very stable in neutral aqueous media, even when irradiated with visible light, which is sufficient for using DCHB as a PDT agent.

We have previously discussed the effects of oxygen and pH values on the photoreactions of

HB with thiol compounds [22]. The results indicated that photothiylation can occur in aerated strong alkaline media [22]. In this present works, we note the effects of oxygen, pH value and organic solvent used on the production of DCHB. The yield of DCHB increases significantly with the oxygen content in the reaction media. We have demonstrated that the presence of oxygen is essential for the formation of compounds A and B [22]. The consistent effect of oxygen on the yield of compounds B and DCHB confirms further the above inference that compound DCHB is derived from compound B. Little DCHB can be formed when oxygen is absent. In addition, the pH value of the reaction media influences the production of DCHB. As the pH value increases, the yield of DCHB increases in a delayed pattern as compared with the conversion yield of HB (Fig. 4). Compounds A and B can be detected at pH 8, but compounds MCHB and DCHB cannot be detected until pH 10. The maximum yields of compound DCHB and HB conversion are both attained at pH 11. In addition, the yield of DCHB is influenced by the organic solvent used. The results are listed in Table 1. It is evident that the facilitation of the production of DCHB occurs when using alcohol-buffer (1:3 by volume, pH 11) as reaction media; the reason for this remains unclear.

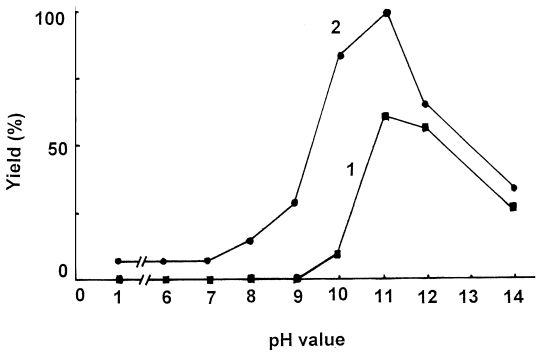


Fig. 4. Effect of pH value on the yield of DCHB (line 1) and the conversion yield of HB (line 2) when the ethanol-buffer (1:3 by volume) solution containing HB (2×10^{-4} M) and cysteine (0.1 M) is irradiated for 20 min.

Compounds A, B, MCHB and DCHB possess the general photodynamic properties of HB. The semiquinone anion radicals can be formed during irradiation in the absence of oxygen. When oxygen is present, active oxygen species, such as superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($\bullet$OH) and singlet oxygen ($^1O_2$) can be produced via the photosensitization of those derivatives. DCHB especially, as a new photosensitizer, has good water-solubility and strong red absorption and potential photodynamic activity in terms of the type I and type II mechanisms, which qualifies DCHB a promising phototherapeutic sensitizer alternative to Photofrin.

3. Experimental

3.1. Materials

α-Phenyl-*N*-butyl nitrone (PBN) was purchased from Aldrich Chemical Company and used without further purification. Cysteine was obtained from Biotech. Company, the Chinese Academy of Sciences. Organic solvents of analytical grades were purchased from Beijing Chemical Plant (China). Solutions were freshly prepared before irradiation and the pH was adjusted, when necessary, by using the following buffer systems: citric acid–sodium hydroxide buffer (for pH < 6), phosphate buffer (for pH 6–8.5), carbohydrate buffer (for pH 9–10), phosphate-sodium hydroxide buffer

Table 1
Effect of organic solvent used in the reaction media on the yield of DCHB

Solvent	CH_3OH	C_2H_5OH	DMSO	DMF	Acetone	Pyridine
Yield of DCHB (%)	60	60	20	20	30	40

The aerated solvent-buffer (1:3 by volume, pH 11) containing HB (2×10^{-4} M) and cysteine (0.1 M) is irradiated for 20 min with light of wavelength longer than 470 nm.

(for pH 11–12) and sodium hydroxide (for pH > 12). The solutions were purged with argon, air and oxygen, according to the experimental requirements. Merck silica gel G60 containing 1% citric acid was used for thin-layer chromatography (TLC).

Crude HA was prepared by acetone extraction of *Hypocrella bambusae* (B.et Br) Sacc. Lipids were removed by counter extraction with petroleum ether. Further purification was carried out on a silica gel column, followed by 1% potassium dihydrogen phosphate-silica gel thin-layer chromatography and recrystallization from acetone twice. HB was prepared by quantitative potassium hydroxide dehydration of HA, followed by neutralization with 10% hydrochloric acid, chloroform extraction, evaporation under reduced pressure and recrystallization from benzene-petroleum ether.

3.2. Measurements

UV-Vis absorption spectra were conducted on a Hewlett–Packard 8541A diode array spectrometer. IR absorption spectra were recorded on a BIO-RAD FTS165 FT-IR spectrometer. ^{1}H-NMR spectra were run on Varian XL-400 (300 MHz) in deuterated water with tetramethylsilane as the internal standard. Mass spectra were performed with FAB MS AEl-MS 50 Kratons spectrometer.

3.3. Methods

All of the photolysis experiments were performed at 20°C, using a 450 W medium-pressure sodium lamp with an external flowing water jacket as light source. A glass long-pass filter was used to cut off light of wavelength shorter than 470 nm. A typical reaction system consisting 4 ml ethanol-buffer (1:3 by volume, pH > 10) solution of a mixture of HB (5×10^{-4} M) and cysteine (0.1 M) was used. The mixture was put in several Pyrex test tubes (1.2×10 cm) and irradiated for 20 min. The irradiated solution was left in the dark for about 20 min with oxygen purging into it. The solution was then acidified with 10% hydrochloric acid and extracted with chloroform. The compounds in the chloroform layer have been previously separated and characterised [22]. Herein, we take the water layer, followed by washing four times with ethyl acetate. The crude residue was purified by Sephadex G-15 column chromatography using water as eluent. The appropriate eluate was collected, and the desired product, i.e. di-cysteine substituted hypocrellin B (DCHB), was obtained in a yield of ca. 60% (60 mg) on the basis of the amount of HB used (100 mg), after evaporating the solvent.

3.4. Compound DCHB

UV-Vis (logε) (in water): 580 nm (4.02), 684 nm (3.95); IR (KBr disk): 3433, 1680, 1599, 1526, 1398, 1284 cm^{-1}; ^{1}H-NMR (300 MHz) (in D_2O) δ: 4.78 (m, 2H, 2NCH), 4.18 (s, 3H, OCH_3), 4.14 (s, 3H, OCH_3), 3.69 (s, 3H, OCH_3), 3.64 (s, 3H, OCH_3), 3.99, 3.48 (dd, 2H, $J = 12$ Hz. 13-H), 3.38 (m, 4H, $2SCH_2$), 2.38 (s. 3H, $COCH_3$) and 1.95 (s, 3H, CH_3). MS (FAB): 774 (M^+).

Acknowledgements

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