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Towards a consensus structure of hypericin in solution: direct evidence for a single tautomer and different ionization states in protic and nonprotic solvents by the use of variable temperature gradient ^{1}H NMR

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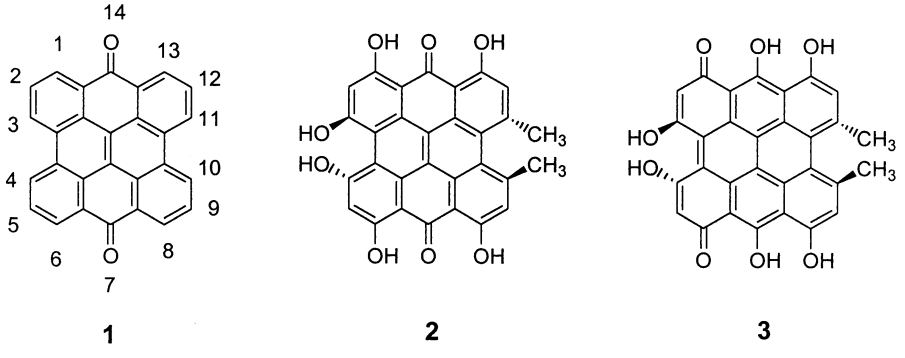
Abstract—Hypericin, a *meso*-naphthodianthrone derivative, displays two types of electronic spectra in organic solvents, attributed to the existence of two tautomeric structures. Variable temperature gradient ^{1}H NMR studies demonstrate the occurrence of only one 7,14-dioxo tautomeric form, for the molecule of hypericin in protic and in nonprotic solvents, differing only in the degree of ionization of the 4-hydroxyl group in the bay region.

1. Introduction

Hypericin, a natural compound identified in the plant genus *Hypericum*,[1–3] and produced synthetically,[4,5] has drawn increased attention over the last decade because of its antiviral, antiretroviral,[6–8] and photodynamic properties.[9–11] It is a photodynamic agent possessing light-induced antiviral activity against several enveloped viruses, including murine cytomegalovirus (MCMV),[12] equine infectious anemia virus (EIAV),[13] Sindbis virus,[14] human immunodeficiency virus (HIV)[15] and herpes simplex (HSV).[6] Hypericin activates tyrosine protein kinase (TPK),[16] protein kinase C (PKC),[17] and succinoxidase.[18] The compound has been shown to inhibit the growth of a variety of neoplastic cell types,[19] and it is used not only for photodynamic therapy (PDT), but also for diagnostic applications.[20]

Hypericin is a partially hydroxylated and methylated derivative of the phenanthroperylene quinone chromophoric system **1** (Fig. 1), occasionally named in the literature as '*meso*-naphthodianthrone'. Although the structure of hypericin (HyH) **2**, 10,11-dimethyl-1,3,4,6,8,13-hexahydroxy-*meso*-naphthodianthrone was established 45 years ago (Fig. 1),[21] its detailed molecular structural aspects are still under debate. According to classical work, hypericin has been assigned as the tautomer with the carbonyl groups



1 **2** **3**

Figure 1. *meso*-Naphthodianthrone skeleton-Q[7,14] (**1**), 7,14-dioxo tautomer of hypericin (**2**), and 1,6-dioxo tautomer (**3**).

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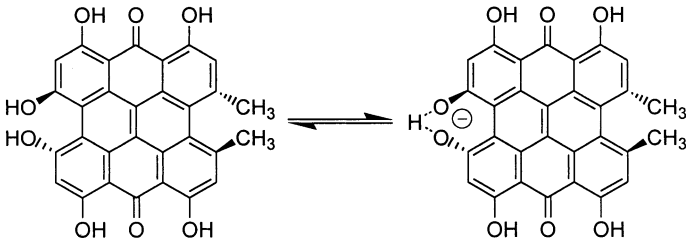

2 **4**

Figure 2. Solvent dependent equilibrium of 7,14-dioxo tautomer of Hypericin HyH, $Q^{7,14}$ (**2**), and its anionic stable form Hy^- (**4**).

in positions 7 and 14 (designated as the tautomer $Q^{7,14}$).[22] Theoretical analysis indicates that hypericin **2** is one out of the 16 structural tautomers possible in principle (10 Kekule, and six nonKekule forms).[23] Utilizing ab initio calculations, the $HyH^{7,14}$ tautomer has been found to be the most stable tautomer, 45 and 83 kJ/mol more stable than the Q,[1,6] and $Q^{1,7}$ tautomers accordingly, considered to be the next most stable forms.[24]

The HyH molecule crystallizes from pyridine in the form of an ion pair of the hypericin monoanion (Hy^-: 3-hypericinate ion), and the pyridinium cation.[25] The facile ionization of **2**, takes place at the OH in the bay region,[†] due to the proximity of the two bay OH groups, one of which is H-bonded to the O atom of the second group in the sterically crowded environment.[26] HyH displays two types of electronic spectrum in solution.[27] The first is observed in protic solvents such as DMSO, methanol, pyridine, TFA, with high absorbance ($\varepsilon \approx 5.3 \times 10^4$), and strong solvatochromic effects, in the range between 600 and 580 nm. The second type is observed in nonprotic solvents including THF, dioxane, ethyl acetate, acetone, and amyl acetate. In these solvents, HyH has its highest absorbency peak reduced, and shifted hypsochromically to 580 nm, exhibiting no solvatochromic effects. The first type has been attributed to the 3-hypericinated anion form **4** (Fig. 2), obtained in these solvents, being identical to that of Hy^-Na^+, formed upon neutralization of **2** with 1 equiv. of NaOH.[25] The second type is attributed to the neutral form **2** (HyH).[25] The neutral form in solution is unstable, and it is converted to the anionic form upon: (a) dilution, (b) addition of protic solvents, or (c) being left in the dark for ca. 48 h.[28] Despite the elucidation of the two forms in various solvents by electronic spectroscopy, efforts to verify their existence by 2D ROESY NMR spectroscopy have thus far resulted in contradictory reports. Initial elucidation of the Hy^-, and HyH forms by spectroscopic means in situ, indicated that the unstable neutral form is the 1,6 dioxo tautomer **3** (Q,[1,6] Fig. 1), whereas the anionic stable form is the 7,14 tautomer **4** ($Q^{7,14}$).[28] However, a recent report concludes that both forms are 7,14-diketo structures ($Q^{7,14}$).[27]

In this article, it is demonstrated, for the first time by recording the variable temperature gradient ^1H NMR spectra, that the same 7,14-diketo structure exists in both protic and aprotic solvents, differing only in the degree of ionization of the 3-OH in the bay region (neutral form **2**, and anionic form **4** accordingly).

2. Results and discussion

Elucidation of the conformational/tautomeric properties of this polycyclic quinone **2** in different solvents, is important for interpreting its photophysical behavior in the excited state, responsible for the photodynamic and biological action of the molecule. Experiments to investigate the structure of hypericin in solution could be designed in principle either in a way to freeze the tautomeric state of the molecule or to obtain structural information by spectroscopic means (including vibrational spectroscopy,[29] ^1H or ^{13}C NMR,[30] and 2D ROESY NMR[27,28,31] spectroscopy) in situ.

Initial investigations using temperature dependent ^1H NMR and 2D ROESY in DMSO-d_6 and in acetone-d_6, concluded that hypericin exists in both solvents as a single tautomer of the neutralized structure **2**, even at temperatures as high as 333 K (or at least that multiple conformers or tautomers cannot be detected by means of NMR spectroscopy).[31] Significant ^1H NMR spectral changes were not observed in the two solvents, and in the temperature range from 240 to 333 K. Using 2D ROESY NMR, correlation of the 9,12-CH signal, with the 10,11-CH_3 signal was detected. This concluded the existence of only one tautomer HyH, Q,[7,14] in both solvents (structure **2**) and gave a partially correct assignment of the ^1H signals. However, it did not address the differences observed in the electronic spectra of the molecule in the two solvents used. Furthermore, the assignment of the bay hydroxyls OH-3,4 based on a broad line at 8.1–8.3 ppm in DMSO was not correct, and led to an incorrect hypothesis about the ionization state of the molecule in this protic solvent (see our discussion below).

In another article, the spectra of hypericin in (DMSO)-d_6, and in (THF)-d_8 were measured using GE 2D ROESY NMR, and the hydroxyl OH-8,13 protons were assigned to the 14.2 ppm signal, and the OH-1,6 protons to the 14.8 ppm signal.[28] In DMSO, the ionic structure of the $Q^{7,14}$ form Hy^- was correctly assigned (structure **4**). The broad signal around 18 ppm for the one bay-hydroxyl proton was identified, and assigned accordingly. However, in THF, mainly due to the lack of correlation of the aromatic protons CH-2,5 (at 6.5 ppm) with any other proton, the wrong tautomeric structure of the neutral $Q^{1,6}$ form was assigned (structure **3**). In this latter case in THF, identification of the bay-hydroxyl protons was not reported.

[†] The positions 3,4 and 10,11 are denoted as bay regions, and those in positions 6,7,8 and 1,13,14 as *peri*-regions.

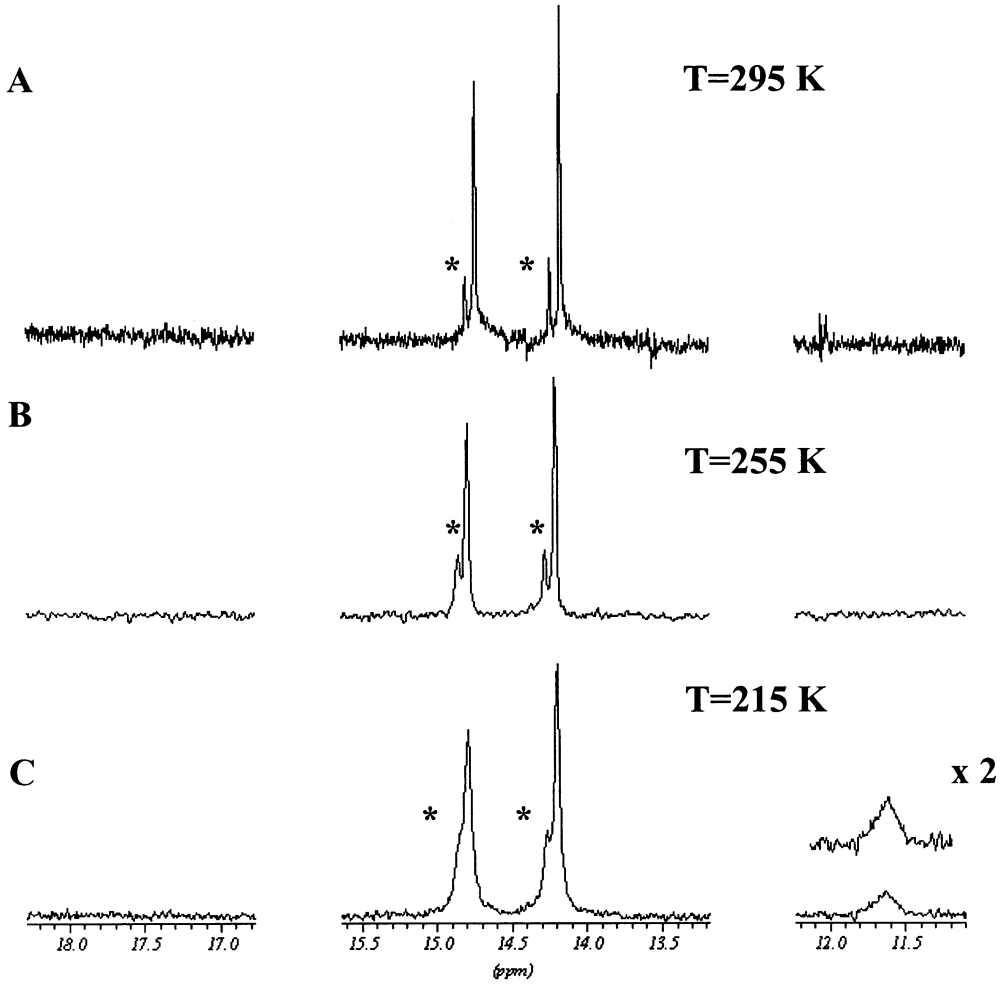

Figure 3. Variable temperature ^{1}H NMR spectra of hypericin in acetone-d_6 (neutral form **2**): number of scans 128, 754 and 1952 for A, B and C, respectively. The asterisk denotes an unknown compound.

In a recent article, clarification of the structural forms of hypericin in protic and nonprotic solvents was conducted, utilizing electronic spectroscopy, 2D gradient-enhanced heteronuclear multiple-quantum correlation (HMQC) and gradient enhanced ROESY.[27] In this work, correlation of the aromatic protons CH-2,5 with OH-1,6 was observed in the nonprotic solvent THF as well as in DMSO, leading to the conclusion that both forms of hypericin in protic and nonprotic solvents are 7,14-diketo ($Q^{7,14}$) tautomeric structure(s), and that other factors are responsible for the difference in the structures of the two compounds. Electronic spectroscopy studies concluded that the unstable form, in nonprotic solvents like THF, has acidic properties and therefore possesses two free OH groups at C_3 and C_4 in the bay region of the molecule.[27] However, no attempts were made to identify these OH protons.

In the present article, recording temperature gradient ^{1}H NMR spectra of hypericin at various temperatures 215–295 K in acetone-d_6, and at 215–295 K in MeOH-d_3, we have been able to unequivocally assign the acidic form of hypericin, existing in nonprotic solvents, to the neutral 7,14-diketo structure **2** ($Q,^{7,14}$ HyH), and the stable form, existing in protic solvents, to its anionic analog 7,14-diketo structure **4** ($Q,^{7,14}$ Hy^-).

In acetone-d_6, the ^{1}H NMR spectrum recorded at room temperature displayed five signals: one methyl (at 2.7 ppm), two aromatic (at 6.7 and 7.4 ppm), and two hydroxyl (14.2 and 14.8 ppm), attributed to the hydrogens of **2**. No signal(s) for the bay hydrogen(s) were recorded. These hydrogen(s), due to the strong acidity are involved in a fast exchange process and therefore are commonly not detected in the ^{1}H NMR spectrum of **2**. However, at low temperatures, the exchange process slows down, allowing the recording of the OH-3,4 protons. Indeed, at low temperatures, a broad line at δ=11.6–11.8 ppm, corresponding to the integral of approximately two protons, was detected, and was assigned to the two bay hydroxyls of the neutral form 2 (Fig. 3). The 3 ppm shift of the bay OH-3,4 signal (at 11.5 ppm), as compared to the signals of the other hydroxyl groups of the molecule (around 14.5 ppm), is anticipated due to the weaker hydrogen bonding interaction of these two 'bay' hydroxyl groups.

The spectrum of hypericin recorded at room temperature in MeOH-d_3, a protic solvent causing the same UV solvachromic effect to that of DMSO-d_6, displayed the five signals described above in acetone solution. The differences in the positions of the signals were not significant (see Section 3). In this case, the spectra recorded at low

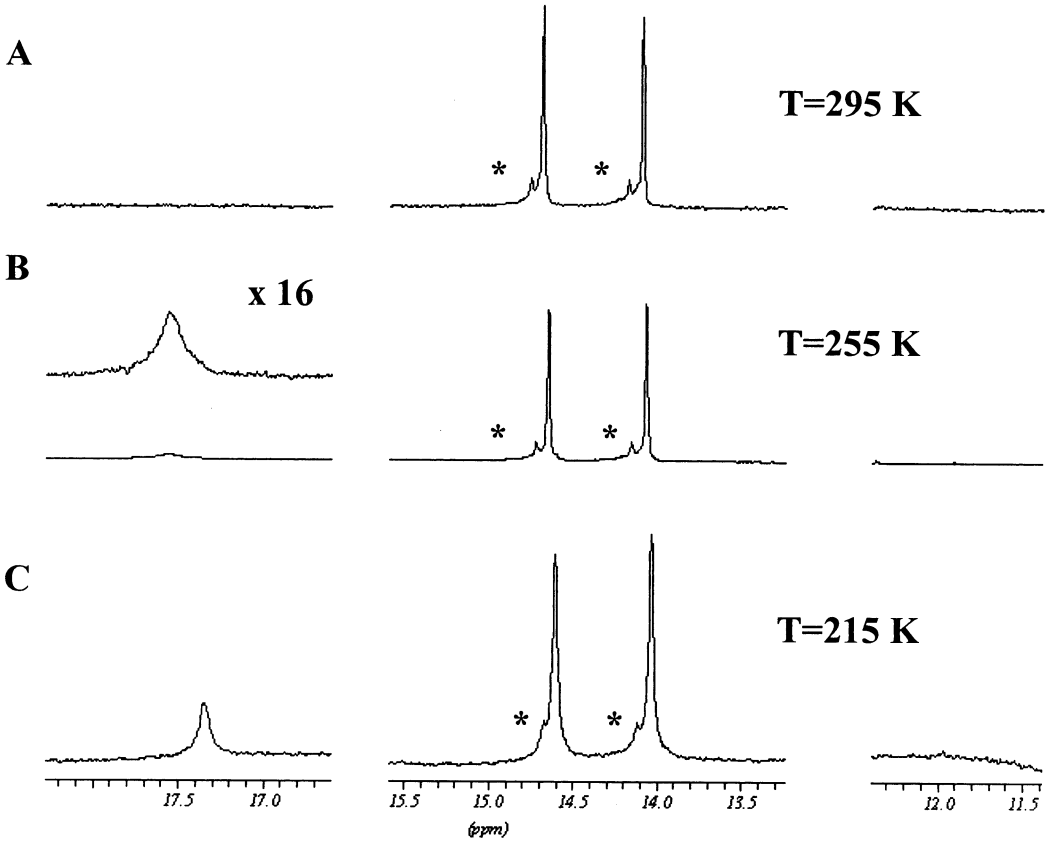

Figure 4. Variable temperature gradient ^{1}H NMR spectra of hypericin in MeOH-d_3 (anionic form **3**): number of scans 64, 762 and 1312 for A, B and C, respectively. The asterisk denotes an unknown compound.

temperatures did not produce the broad peak, expected for the two bay OH-3,4 protons, around 11.5 ppm. Instead a broad peak at δ=17.3–17.5 ppm, corresponding to the integral of one proton, was recorded, and was assigned to the OH-3 of the 4-hypericinate ion, leading thus to the assignment of the 4-hypericinate ionic form of **4** (Fig. 4). This peak has previously been recorded, and correctly assigned in the spectrum of hypericin in DMSO-d_6,[28] however, this is the first time that such a resonance has been observed in a protic solvent like MeOH-d_3. This is due to the utility of the WATERGATE pulse sequence for gradient[33,34] which does not eliminate fast exchange of the OH resonance with the solvent. The broad line at 8.2 ppm reported earlier in DMSO-d_6,[31] and assigned to the bay hydroxyl protons, has not been recorded either in our spectra (in acetone-d_6 or in MeOH-d_3), or in the spectrum of others (in DMSO-d_6).[28] This means that the specific signal was generated in the specific spectrum either by an impurity, or by an artifact.

The existence of one single structure in both types of solvents, is also supported by the fact that the positions of the other groups in the aromatic skeleton of hypericin remain the same in both solvents (see Section 3). The singlets at 14.1 and 14.7 ppm, corresponding to the four hydroxyl groups, are recorded at the same position in both solvents, even at low temperatures (Figs. 3 and 4). This means that the hydroxyl groups remain attached to the same carbons (at the 8,13 and 1,6 positions), regardless of the solvent in which the molecule is dissolved. The same is also true for the methyl groups (at the 10,11 positions) which exhibit a single peak at 2.7 ppm in both solvents.

In conclusion, the confusion regarding the structural forms of hypericin, in various solvents has been solved. Our findings are in agreement with the theoretical analysis performed at the RMP2/6-31G(d) level of theory, which rationalizes the spectra and the excited-state kinetics of hypericin due to the deprotonation of the bay hydroxyl groups.[34] The same analysis predicts that a bay hydroxyl can be deprotonated in the ground state, which leads to the existence of two possible structural isomers (un-ionized, and ionized), in different solvents. These two isomers are separated by a very large inversion barrier.

3. Experimental

3.1. General

Hypericin was purchased from Fluka (Buchs, Switzerland) and used without further purification. ^{1}H NMR spectra were measured in anhydrous MeOH-d_3 and acetone-d_6 (Aldrich) with a Bruker AMX-400 spectrometer equipped with a z-gradient unit.[32,33] The suppression of the OH resonance of MeOH-d_3 was achieved with the use of WATERGATE pulse sequence for gradients. The concentrations were ca. 1 mg/ml. Chemical shifts are reported relative to internal Me_4Si (δ=0.000) of known concentration. Data were processed using UXNMR and WINNMR (Bruker) software.

The number of scans (64–1952) was dependent on the concentration which is reduced at low temperatures. 1D ^{1}H NMR spectra of hypericin were recorded at 295, 280, 260, 255, 240, 235, 220, 215, 205, 215 K in acetone and 295, 285, 275, 265, 255, 245, 235, 225, 215 K in methanol. Hypericin samples are reported to decompose upon prolonged exposure to ambient light and were kept in the dark and at low temperature.

The recorded spectra at room temperature are as follows:

3.1.1. Hypericin (2). ^{1}H NMR (400 MHz, acetone-d_6) δ 14.75 (2H, s, C1, C6–OH), 14.17 (2H, s, C8, C13–OH), 11.75 (2H, br s, C3, C4–OH), 7.39 (2H, s, C9, C12–H), 6.70 (2H, s, C2, C5–H), 2.78 (6H, s, C10, C11–CH_3).

3.1.2. 4-Hypericinate anion (4). ^{1}H NMR (400 MHz, MeOH-d_3) δ 17.37 (1H, br s, C3–OH), 14.68 (2H, s, C1, C6–OH), 14.09 (2H, s, C8, C13–OH), 7.27 (2H, s, C9, C12–H), 6.56 (2H, s, C2, C5–H), 2.71 (6H, s, C10, C11–CH_3).

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