Pergamon

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Tetrahedron Letters 44 (2003) 6285–6288

TETRAHEDRON LETTERS

Identification of isopseudohypericin, a new phenanthroperylene quinone obtained by the alkaline treatment of pseudohypericin

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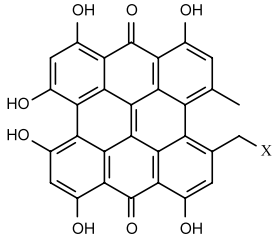
Received 6 June 2003; revised 18 June 2003; accepted 18 June 2003

Abstract—At high pH, pseudohypericin from St. John's Wort (*Hypericum perforatum*) is converted into a single product which has been isolated. LC–MS analysis indicated that the molar mass of this product is identical to that of pseudohypericin. ^{1}H and ^{13}C NMR analysis indicated that the compound is a novel phenanthroperylene quinone which we have named isopseudohypericin; it is a cyclic ether which is an isomer of pseudohypericin.

Pseudohypericin (**1**) and its dehydroxylated analog hypericin (**2**) are phenanthroperylene quinones (Fig. 1) which are highly specific to St. John's Wort (*Hypericum perforatum*), a plant which has been exploited since ancient times for its numerous therapeutic activities.[1] Recently, this plant has come into the scope of scientific medicine notably for its therapeutic activities in the treatment of mild depression[2], and its antiviral activity.[3] An exhaustive review about the chemistry of this 'fascinating group of natural products' is now available due to the compilation work of Falk.[4]



1 : X = OH
2 : X = H

Figure 1. Chemical structure of pseudohypericin (**1**) and hypericin (**2**).

Cameron[5] previously reported that **1** is unstable in an alkaline environment and he proposed **3** as the structure of the product. In the presence of strong acid (H_2SO_4), he observed that **2** is converted into cyclopseudohypericin **4**. This phenanthroperylene quinone (already mentioned in 1952 by Brockmann,[6] the discoverer of hypericin) can be definitively identified by virtue of its ^{1}H NMR spectrum. More recently, Wirz[7] reported that **1** is unstable even in relatively mild storage conditions, proposing that cyclopseudohypericin may be generated from **1**. Häberlin[8] and then Gaedeck[9] used HPLC to detect a novel phenanthroperylene quinone in extracts of the plant and, on the basis of UV–vis spectra recorded using a diode array detector, attributed to it structure **4**. Häberlin also described the synthesis of **4** from **2** in an alkaline environment (MeOH–ammonia). In the course of investigations into the preparation of extracts of the plant,[10] we found **1** to be unstable at high pH (whereas in similar conditions, **2** was simply ionized without any structural change).

The structure of cyclopseudohypericin (Fig. 2) has not been definitively established, and the term is currently used for both a saturated compound (**4a**: Chemical Abstracts Registry Number 61350-17-2) and an unsaturated one (**4b**: a more oxidized species recently mentioned by Poutaraud;[11] Registry Number 54161-41-0).

In preliminary work, we generated this putative compound by the alkaline treatment of **1**. We sought to

Keywords: *Hypericum perforatum*; pseudohypericin; isopseudohypericin; new phenanthroperylene quinone.

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0040-4039/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0040-4039(03)01527-2

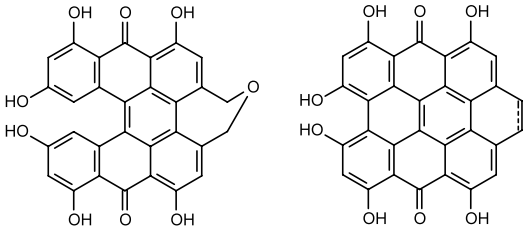


Figure 2. Proposed structures for cyclopseudohypericin.

investigate the phenomenon more deeply with tightly controlled experimental conditions and using pseudohypericin which we have purified in quantity in the laboratory.[12]

The key experiment was the following: 10 μl of 0.5N NaOH was added to 3 ml of a 20 mg/l solution of **1** in 80% EtOH. This was carried out directly in a quartz cuvette and the UV–vis spectrum between 250 and 750 nm was recorded at regular intervals. The spectrum (Fig. 3) was observed to change steadily from that of **1** (spectrum A) to that of a novel compound (spectrum C). Acidification of the reaction medium by the addition of 20 μl of 0.5N HCl resulted in the appearance of spectrum D. Successive additions of either NaOH or HCl caused switching between spectrum D and spectrum C.

HPLC analysis of both the alkaline and acidic mixtures revealed a single peak with a retention time slightly longer than that of **1**. The UV–vis spectra of the peaks, obtained with a photodiode array detector are identical to spectrum B (shown in Fig. 2). These results indicate that, in an alkaline environment, **1** is converted into a single compound, which we call isopseudohypericin (**5**); the spectrum of this compound is pH-dependent. This compound was isolated and its structure was determined. Vacuum drying of the reaction medium—either acidic or alkaline—failed to yield a characterizable compound and ultimately **5** was obtained by solid–liquid extraction. **1** Was converted to **5** by treating 50 mg of **1** in 50 ml of 80% EtOH in the presence of 1 ml of 0.5N NaOH. On completion of the reaction, the medium was diluted with 50 ml of water and run over a C18-conjugated silica column (Chromabond®). The column was washed with 500 ml of water and then with MeOH. The colored fraction was recovered and treated in a vacuum evaporator to yield 35 mg of **5** in the form of a black powder. UV–vis spectra of **5** were recorded in a quantitative fashion (20 mg/l) in MeOH and MeOH containing either NaOH or HCl. The three profiles were identical to those shown in Figure 2, indicating that the isolation process had not affected the structure of **5**. Corresponding values for λ_{max} and ε (derived for a molar mass of 520 D) are as follows: λ_{max} ($\varepsilon_{max}\times10^3$) MeOH/NaOH (C): 591 (10.1); 436 (10.4); 355 (28.6); 238 (45.2). MeOH (B): 560 (10.7); 439 (10.0); 344 (23.6); 238 (41.1). MeOH/HCl (D): 551 (1.7); 436 (9.0); 338 (23.3); 238 (38.2).

HPLC analysis of **5** showed a single, homogenous peak which gave with photodiode array detector UV–vis spectrum D. LC–MS analysis was first carried out on a mixture containing pure samples of both **1** and hypericin **2** in order to validate the method. Given that the analyzed molecules were negatively ionized, the results were as expected (giving a molar mass of 519 for **1** and 503 for hypericin). Parallel analysis of **5** gave a definitive molar mass of 519. No peak corresponding to a molar mass of 501 could be detected on the chromatogram thereby ruling out the possibility that **1** was being converted into cyclopseudohypericin **3** or **4**. However, structure **3** as proposed by Cameron[5] could not be ruled out (Fig. 2).

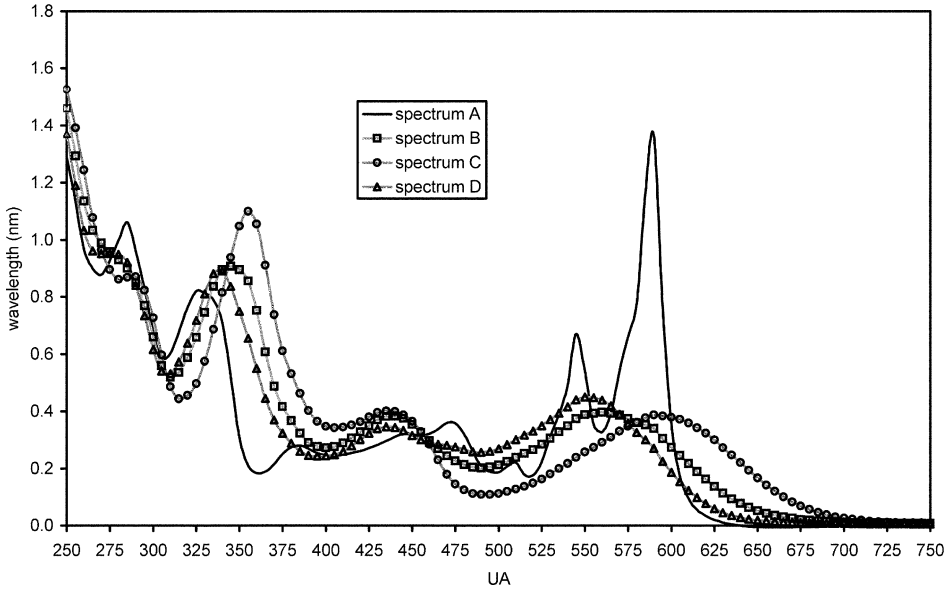
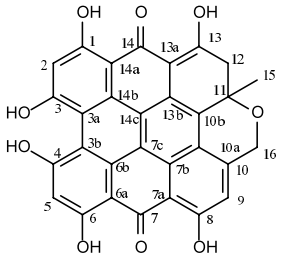


Figure 3. UV–vis spectra of **1** in MeOH (A) and **5** in MeOH (B) with NaOH (C) and HCl (D).



5

Figure 4. Chemical structure of isopseudohypericin (**5**).

Structure of isopseudohypericin **5** (Fig. 4) was deduced from the ^{1}H and ^{13}C NMR spectra recorded in DMSO and by HMBC correlations. The proton spectrum reveals the presence of four distinct phenolic proton signals (compared with just 2 in **1**), and only 3 benzene proton signals (compared with four in **1**). The signal from the methyl group (C15) observed at 2.66 ppm in **1** is shifted to 1.64 ppm in **5**; the signals due to the AB system (4.65 and 5.1; $J=13$ Hz) attributed to the two H16 hydrogen atoms in **1** are shifted to 5.3 and 5.54, $J=16.3$ Hz in **5**. Finally, an extra AB system is observed in **5** at 2.83 and 3.28 ppm with $J=14.8$ Hz; this is attributed to the two H12 hydrogen atoms. The NMR data were the following.

^{13}C (75.5 MHz), **^{1}H** (300 MHz) NMR and *HMBC* correlations data of **5** (DMSO-d_6, TMS standard): C1: 165.02; **14.86**. C2: 108.21; **6.34**; *1*, *3*, *14a*, *3a*. C3: 173.85, *2*. C3a: 117.42, *2*. C3b: 115.1, *5*. C4: 173.63, *5*. C5: 107.21; **6.69**; *4*, *6*, *3a*, *6a*. C6: 167.31; **14.35**. C6a: 104.65. C6b: 126.78. C7: 183.35. C7a: 109.08. C7b: 126.12. C7c: 121.14. C8: 172.36; **15.69**. C9: 113.45; **7.33**; *8*, *10a*, *16*. C10: 142.21. C10a: 116.28. C10b: 125.58. C11: 74.91. C12: 52.06; **2.83–3.28**; $J=14.8$ Hz; *11*, *13a*, *12b*, *15*. C13: 172.46; **16.68**. C13a: 105.15. C13b: 125.36. C14: 183.62. C14a: 104.81; *2*. C14b: 126.78. C14c: 121.14. C15: 64.25; **1.67**; *11*, *12 10b*. C16: 22.32; 5.30–5.54; $J=16.3$ Hz; 11, 10a, 10.

The fluorescence spectra of **1** and **5**, recorded in MeOH ($\lambda_{ex}=591$ nm) display fluorescense band at 594 and 637 nm and 591 and 637, respectively. Values obtained for **1** are in good agreement with those (595 and 640 nm) given for hyperin **2**.[4]

As a first approximation, the conversion to **5** can be explained through the formation of a C16 alkoxide at high pH. The tautomeric forms of hypericin **2** have been analyzed on a theoretical basis by Etzlstorfer and Falk[13] but not those of **1**. The complexity of the situation makes it difficult to deduce mechanisms but, using UV–vis spectroscopy, we studied the effect of NaOH concentration on the rate of the reaction. We used a 0.03 mM solution of **1** ($\approx$15 mg/l) in 80% EtOH to which were added aliquots of NaOH solution to give final concentrations of 1.0, 0.5, 0.2 and 0.1 mM. All operations were conducted at 20°C. UV–vis spectra were recorded over time and used to determine the half-time for the conversion of **1**: the results were $\approx$10, 30, 50, 7200 min, respectively. In the absence of NaOH, **1** was stable for three weeks at room temperature.

An enormous increase in the rate of the reaction is consistently observed when the NaOH concentration is increased from 0.1 to 0.2 mM. This could be interpreted in terms of the presence of six ionizable sites on **1** corresponding to 0.18 mequiv. This suggests that the conversion of **1** into **5** occurs with the phenol groups becoming ionized prior to ionization of the hydroxymethyl group. The two hydroxyl groups at the *bay* region (C3 and C4) are readily ionized ($pK_a=1.8$) and are completely dissociated even in highly diluted polar solvents. Ionization of the four others (in the *peri* region) is similar to that of the common phenol group with pK_a values of $\approx$9 and 12.[14] **5** would be generated from the dissociated form of **1** by an intramolecular Michael addition of the C16 alkoxide followed by re-protonation (as outlined in Fig. 5) In spatial terms, the two, 'propeller' or 'double-butterfly',[15] conformers of **1** would be susceptible to permit the attack by the C16 oxygen atom at C11 perpindicularly to the plane of the methylated ring.

The conditions for the formation of **5** from **1** are currently under investigation, as is the relationship between **5** and the phenanthroperylene quinone detected in native plant material.

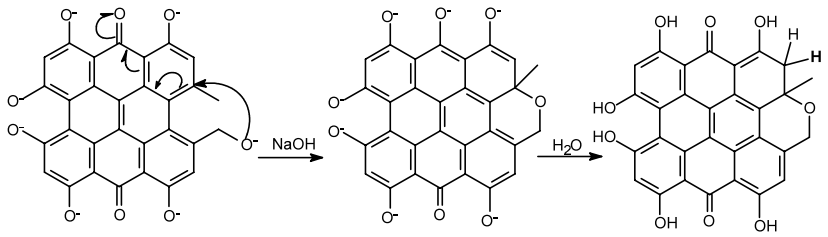


Figure 5. Proposed mechanism for transformation of **1** into **5**.

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